ISSN 1996-0700

Asian Journal of Biotechnology

Knowledgia SCIENTIFIC
A Place to Publish Outstanding Research

http://knowledgiascientific.com

Asian Journal of Biotechnology 6 (1): 1-14, 2014
ISSN 1996-0700 / DOI: 10.3923/ajbkr.2014.1.14

Optimizations of Laboratory-scale Vacuum-assisted Agroinfiltration for Delivery of a Transgene in *Nicotiana benthamiana*

Hwei-San Loh and Samson Baranzan Wayah
School of Biosciences, Faculty of Science, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor, Malaysia

Corresponding Author: Hwei-San Loh, School of Biosciences, Faculty of Science, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor, Malaysia Tel: +060389248215 Fax: +060389248018

ABSTRACT

Agrobacterium-mediated transient gene transfer or agroinfiltration is a method to induce transient expression of heterologous proteins in plants. It offers speed, low cost and convenience when compared to traditional plant transformation. This study was aimed to set up a simple but feasible laboratory-scale vacuum agroinfiltration system. Basically, the procedures involved whole plant immersion into transformed *Agrobacterium tumefaciens* suspension and pressure application via vacuum drawing the agrobacteria into the plant tissues homogeneously. Optimizations of its parameters such as vacuum pressure and treatment duration towards the expression activity of a reporter, Green Fluorescence Protein (GFP) were conducted. The transformed *A. tumefaciens* strain LBA4404 carrying the recombinant plant viral vector, pEAQ-HT-GFP was infiltrated into the leaves of *N. benthamiana* via vacuum mode. In general, vacuum pressure and duration of infiltration had significant effects ($p<0.05$) on transient expression of GFP. Both fluorescence microscopy and Western blotting showed that vacuum agroinfiltration at 600 mmHg for 3 min had the highest and 400 mmHg for 2 min had the lowest mean GFP expression levels. This modified vacuum agroinfiltration approach utilized only distilled water and *A. tumefaciens* as components of the infiltration solution had substantially reduced the chemical cost. In conclusion, a simple and cost-effective laboratory-scale vacuum-assisted agroinfiltration system has been developed for recombinant protein production *in planta*.

Key words: Vacuum agroinfiltration, *Nicotiana benthamiana*, *Agrobacterium tumefaciens*, transient expression, green fluorescence protein

INTRODUCTION

Global demand for therapeutic recombinant proteins and high value products including vaccines, antibodies, diagnostic proteins, secondary metabolites and industrial enzymes can no longer be solely supported by the use of traditional systems which has necessitated the search for more sustainable systems. Traditional systems including mammalian cell cultures and bacteria which incur high production cost particularly from the downstream processing and requirement of sophisticated equipment for fermentation (Ma *et al*., 2003). Plants on the other hand have several advantages over traditional systems such as relatively simple and inexpensive growth requirements which make scaling up almost unlimited and cheap, chance of pathogen contamination is low and eukaryotic protein modifications which include disulphide bridging and glycosylation required by mammalian proteins for performing their biological functions can occur (Menassa *et al*., 2001).

Nicotiana benthamiana is one of the major plants used as biofactories mainly due to its amenability to genetic modifications and established growth and harvest procedures. The fact that *N. benthamiana* is leafy, has high amount of biomass and soluble proteins makes it a suitable plant for recombinant protein production. *N. benthamiana* has been used for transient and stable expression of recombinant proteins via infection with either *Agrobacterium* or plant virus. It is not consumed, has hence reduced the chances of contamination to the feed and food chains by transgenic materials (Tremblay *et al*., 2010).

One of the widely used methods for transient expression studies is agroinfiltration or *Agrobacterium*-mediated transient gene delivery. Agroinfiltration can be administered through direct syringe or vacuum inoculation. Direct syringe infiltration described by Natorajan *et al*. (2010) as injecting genetically-engineered agrobacteria carrying desired transgene into plants using a syringe without needle. When gentle pressure is applied, agrobacterial suspension is delivered into a leaf via stomata. However, this technique suffers from the lack of homogeneous gene expression. Thus, it necessitates the development of vacuum agroinfiltration that can produce homogeneous and high transient expression (Sainsbury *et al*., 2010; Sainsbury and Lomonossoff, 2008). Vacuum-assisted infiltration delivers agrobacteria into a variety of plant tissues such as leaf discs, intact leaves or whole plants by submerging the target tissues into the agrobacterial suspension. Application of vacuum to the plants forces air out from stomata at the first place. When vacuum is released, the agrobacterial suspension is drawn into plant tissues due to pressure difference (Tague and Mantis, 2006). This method is suitable for large scale production of recombinant proteins by offering advantages in terms of speed, yield and cost (D'Aoust *et al*., 2009). In fact, the combination includes three biological systems made of virus, bacteria and plant, is the best to overcome shortcomings in transient expression system. This is attributed to the rapid expression from virus, vector efficiency and long distance movement by *A. tumefaciens* as well as post-translational capabilities and low production cost of plants (Gleba *et al*., 2005). With all the offered good qualities, recombinant proteins can be produced at high level, fast, cheap and safe; it is indeed an ideal option for industrial mass production (Gleba *et al*., 2005). Vacuum agroinfiltration has currently been employed extensively on a commercial scale for recombinant protein production by biopharmaceutical industries. One of them is Fraunhofer USA-Center for Molecular Biotechnology (Yusibov and Mamedov, 2010) which has used this platform to reduce cost of production and consequently makes the biopharmaceutical products cheaper and more affordable to needy.

The industrial scale vacuum agroinfiltration or 'magnifection' (Gleba *et al*., 2005) technologies have been innovatively implemented by a few established pharmaceutical manufacturers but the laboratory-scale system is scarcely described. Plant-based pharmaceuticals are still a young area to explore, so, establishment of a simple, cost effective but feasible laboratory-scale vacuum agroinfiltration system is important for facilitating this kind of *in planta* expression studies. In fact, there have still been many ground-breaking researches sprouted from small laboratories or academic institutions which have basic but sufficient facilities. So, this study describes an in-house set-up of laboratory-scale vacuum agroinfiltration, optimizations of its operational and reagent conditions in order to acquire high protein expression of plants in a low cost process. This system also confers minimal harm to the plants which serving as a healthy biofactory for high value products.

MATERIALS AND METHODS

Plant viral vectors: Two plant expression vectors engineered from cowpea mosaic virus, namely the pEAQ-HT (an empty vector to serve as negative control) and pEAQ-HT-GFP (a recombinant vector expressing the reporter, Green Fluorescence Protein or GFP) by Sainsbury *et al.* (2009a) were used for studying the modified vacuum agroinfiltration system and optimizations of its parameters. Both pEAQ-HT and pEAQ-HT-GFP were transformed into *Agrobacterium tumefaciens* strain LBA4404 using electroporation method. The resultant transformants were named as Ag/pEAQ-HT and Ag/pEAQ-HT-GFP thereafter.

Plant growth condition: All *Nicotiana benthamiana* plants were grown under greenhouse condition in a soil mixture containing coco peat, sand and organic top soil in the ratio of 2:1:1 at 24°C under a 9:15 h light-dark cycle before and after agroinfiltration. Consistent and sufficient amount of fertilizer and water were supplied to ensure optimal growth of the plants. Six weeks old plants with similar number of leaves were used in this study.

Preparation of infiltration medium: The transformed agrobacterial cells, Ag/pEAQ-HT and Ag/pEAQ-HT-GFP were grown on separate Luria-Bertani broth containing 28 μg mL^{-1} rifampicin and 50 μg mL^{-1} kanamycin at 28°C for 42 h in an incubator with shaking condition at 220 rpm. The agrobacterial cells were harvested by spinning at 2,000×g for 20 min at 6°C. The agrobacterial pellet was resuspended in autoclaved distilled water to reach different absorbance values of 0.3, 0.6 and 1.0 read at optical density of 600 nm (OD_{600}) using Biophotometer Plus (Eppendorf, Germany).

Agroinfiltration of *Nicotiana benthamiana*: For determining the most appropriate agrobacterial OD_{600} used in the infiltration medium, the abaxial side of fully expanded leaves was pricked several times with a small pipette tip first and the agrobacteria containing-infiltration medium was gently infiltrated into the leaf intercellular spaces through the wound using a 1 mL plastic syringe without needle until the whole leaf became wet.

Meanwhile, watering of plants was only done 24 h prior to vacuum agroinfiltration in order to prevent soil dropping into the infiltration medium. The vacuum agroinfiltration system was attempted in different manners in order to make the setting as simple as possible, cheap but effective for infiltration purpose. Then, vacuum pressure was set to desired levels (600 mmHg for 3 min, 600 mmHg for 2 min, 400 mmHg for 3 min and 400 mmHg for 2 min) and applied to three plant replicates for both negative control (Ag/pEAQ-HT) and GFP (Ag/pEAQ-HT-GFP) treatments. Once agroinfiltration was done, infiltrated plants were watered for a period of 10 days post-infiltration (Dpi). The infiltrated leaves were harvested every 2 Dpi up to 10 Dpi for subsequent analyses.

Fluorescence microscopic observation: Infiltrated *N. benthamiana* leaves were viewed under the stereoscopic zoom microscope model SMZ-1500 (Nikon, Japan) with camera and fluorescence light attachment using a GFP filter (455-485 nm excitation) for fluorescence signal. Microscopic observation was done on three leaves each plant replicate using 10X magnification to determine the GFP expression level which was then calculated to obtain the mean percentage of green fluorescence area per total leaf area by the NIS Element software (Nikon, Japan).

Protein extraction and quantification: Total soluble protein was extracted from 200 mg of infiltrated leaves according to the method described by Sainsbury *et al.* (2009b). The harvested leaves were ground into powder using liquid nitrogen and then resuspended in 2X volume (w/v) of extraction buffer. This was subsequently spun at 15,000×g for 10 min at 4°C. The supernatant was collected and protein quantification was carried out using Pierce 660 nm protein assay reagent (Thermo Scientific, USA) according to manufacturer's instructions.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blotting: Firstly, 12% separating and 7% stacking gel mixtures were prepared. Protein samples amounting 3 μg each were loaded onto the wells. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 150 V for 80 min to separate the total soluble proteins extracted. BenchMark™ pre-stained protein ladder (Invitrogen, USA) and Spectra Multicolor broad range protein ladder (Fermentas, USA) in a volume of 5 μL each were used for SDS-PAGE and Western blotting, respectively. One of the gels was subjected to staining using SimplyBlue™ SafeStain (Invitrogen, USA) for viewing purpose. Another unstained gel was electroblotted onto polyvinylidene difluoride (PVDF) membrane at 25 V for 90 min using XCell II™ blot module (Invitrogen, USA). The blotted membrane was firstly blocked with 4% (w/v) milk in 1X PBS-T buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 0.05% (v/v) Tween-20), then hybridized with 1:10,000 diluted primary rabbit anti-GFP polyclonal antibody (Abcam, UK) and 1:10,000 diluted secondary horseradish peroxidase-conjugated anti-rabbit IgG (Zymed, USA) at room temperature for 2 h each respective step. Three washes in PBS-T buffer were conducted after each step. Next, the membrane was incubated with 3 mL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate until development of desired signal level. Image was captured and protein bands were analyzed using GS800 calibrated imaging densitometer and QuantityOne software (BioRad, USA) in reflective density per mm^2 (RD mm^{-2}).

Data analysis: Completely randomized design was employed for the optimization of vacuum agroinfiltration parameters, namely the vacuum pressure and treatment duration that affecting the transient expression level of GFP. Each parameter was tested at two levels, i.e., vacuum pressure at 400 and 600 mmHg and treatment duration for 2 and 3 min. All data points were based on a mean of three replicates and values obtained were subjected to two-way analysis of variance (ANOVA) by comparing mean separation of least significant difference ($p<0.05$) using SPSS software (version 20.0).

RESULTS

Determination of agrobacterial concentration: Among the three OD_{600} values (0.3, 0.6 and 1.0) of transformed *A. tumefaciens* tested, as shown in Fig. 1, it can be observed that at high absorbance value (OD_{600} = 1.0), severe chlorotic, necrotic and wilting symptoms (Fig. 1a) appeared starting from 6 Dpi. These symptoms declined with lower concentration of *A. tumefaciens* used where moderate leaf chlorosis was found at OD_{600} = 0.6 (Fig. 1b) and the infiltrated plant leaves remained rather healthy at OD_{600} = 0.3 (Fig. 1c). In contrast, all these symptoms were not seen in non-infiltrated leaves (Fig. 1d). Both plants infiltrated with Ag/pEAQ-HT and Ag/pEAQ-HT-GFP showed similar observations.

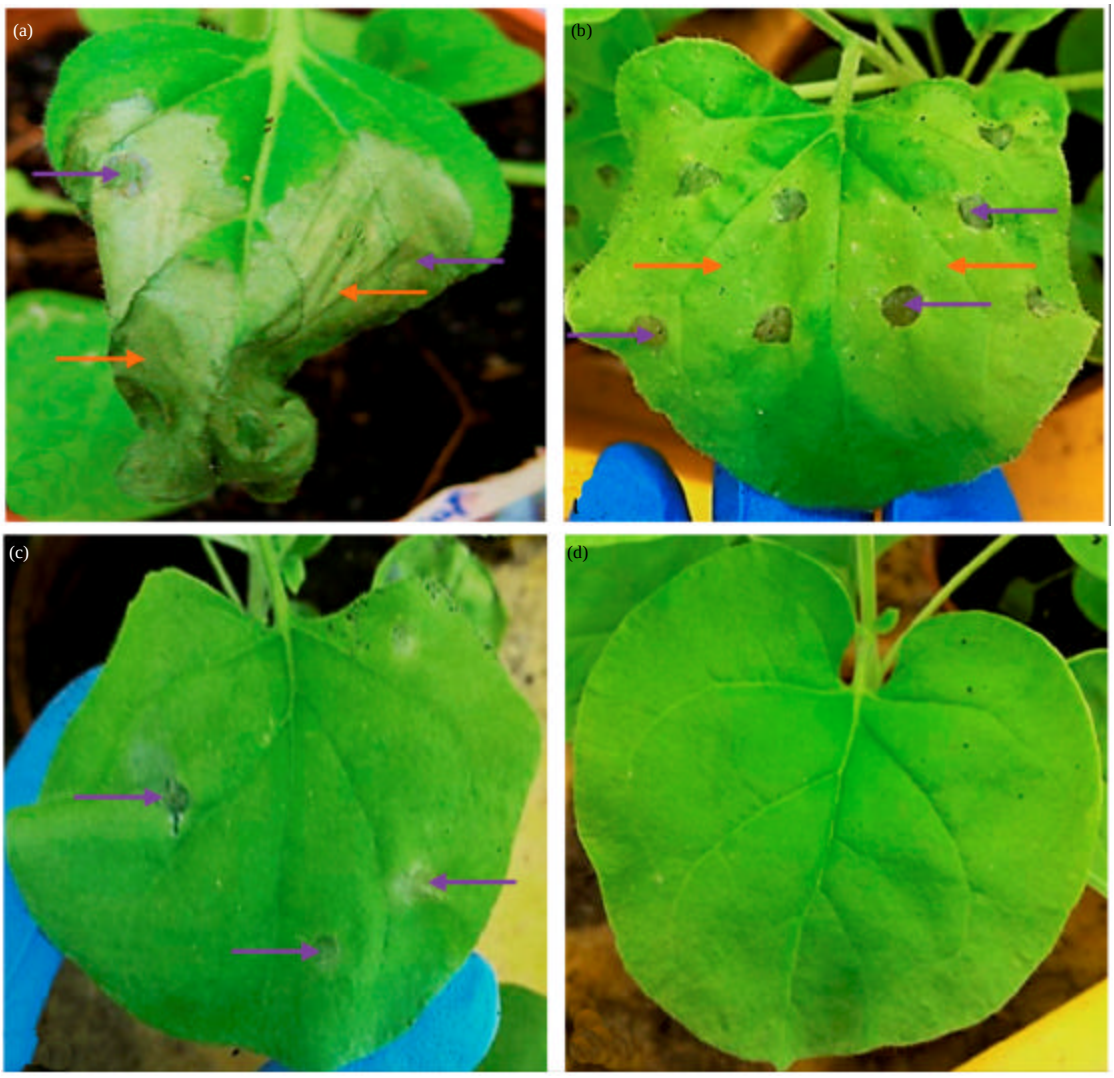

Fig. 1(a-d): Physical appearance of *N. benthamiana* leaves infiltrated with different agrobacterial concentrations (Ag/pEAQ-HT-GFP) at 6 Dpi, (a) OD_{600} = 1.0 caused severe chlorotic, necrotic and wilting symptoms (red arrows), (b) OD_{600} = 0.6 triggered moderate chlorotic symptom (red arrows), (c) OD_{600} = 0.3 produced very mild or almost no chlorotic symptom and (d) Non-infiltrated healthy leaf, purple arrows indicate the physical lesions caused by infiltration using a syringe without needle

Set-up of vacuum agroinfiltration system: The finalized sequential steps involved in setting up the laboratory-scale vacuum agroinfiltration system were shown in Fig. 2. Briefly, the vacuum pump and vacuum jar were set up (Fig. 2a). Fresh infiltration solution comprising autoclaved distilled water and agrobacterial cells at OD_{600} = 0.3 was prepared up to 500 mL in a beaker (Fig. 2b) and beaker containing two spatulas was placed inside the vacuum jar (Fig. 2c). The soil in pot was covered with aluminum foil and the dusts on leaf surface were cleansed (Fig. 2d). Then, the plant was inverted and immersed into the agrobacterial infiltration solution (Fig. 2e) and

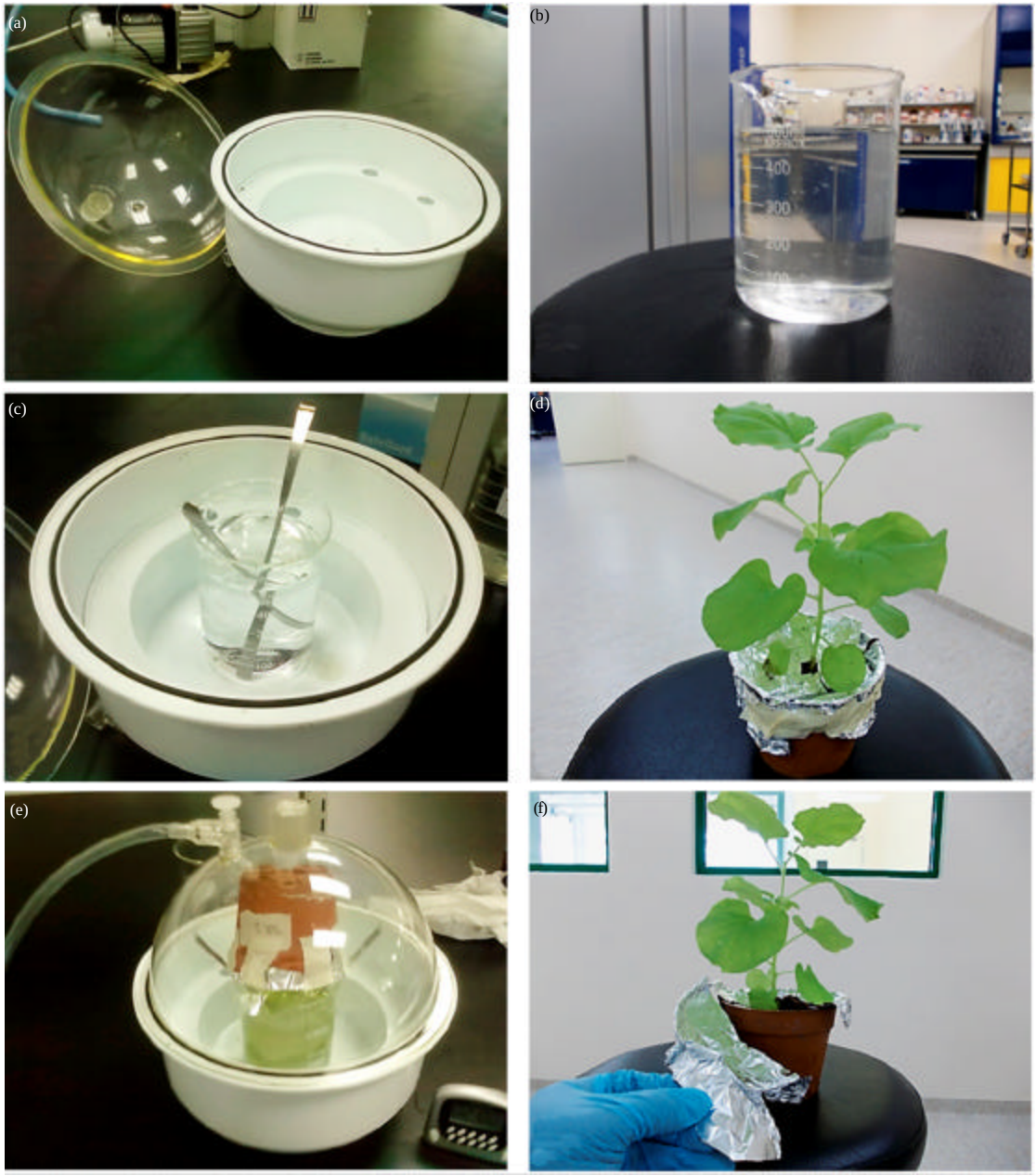

Fig. 2(a-f): Summarized steps of vacuum agroinfiltration system, (a) Setting-up of vacuum pump and vacuum jar, (b) Freshly prepared infiltration solution in a beaker, (c) Placing the beaker containing two spatulas inside the vacuum jar, (d) Wrapping soil with aluminum foil, (e) Vacuum infiltration of plant in an inverted position and (f) Unwrapping of pot after vacuum infiltration

vacuum jar was closed tightly followed by switching on the vacuum pump according to desired treatment parameters. Subsequently, the lid of vacuum jar was opened slowly and the pot was taken out from the vacuum jar and unwrapped (Fig. 2f). The infiltrated plants were allowed to further grow until the time for harvest.

Microscopic observation of GFP: Plants harvested at 2, 4, 6, 8 and 10 Dpi were viewed under stereoscopic zoom microscope and the representative pictures of green fluorescence signals of leaf area (Ag/pEAQ-HT-GFP-infiltrated) are shown in Fig. 3. These fluorescence signals were observed increasing with higher parameters of infiltration conditions applied, i.e., 400 mmHg for 2 min (Fig. 3a) <400 mmHg for 3 min (Fig. 3b) <600 mmHg for 2 min (Fig. 3c) <600 mmHg for 3 min (Fig. 3d) as ranked in an ascending order. In contrast, no similar fluorescence signals were seen in Ag/pEAQ-HT-infiltrated plants.

When the fluorescence level was expressed in percentage per total leaf area (Fig. 4), it was found that treatment at 600 mmHg for 3 min had the highest mean level of 93.7% while mean values obtained for 600 mmHg for 2 min, 400 mmHg for 3 min and 400 mmHg for 2 min were 88.0, 80.0 and 44.0%, respectively at 10 Dpi. In general, all four vacuum infiltration conditions showed a time-dependent increment on the fluorescence level and mean values obtained were significantly different ($p<0.05$).

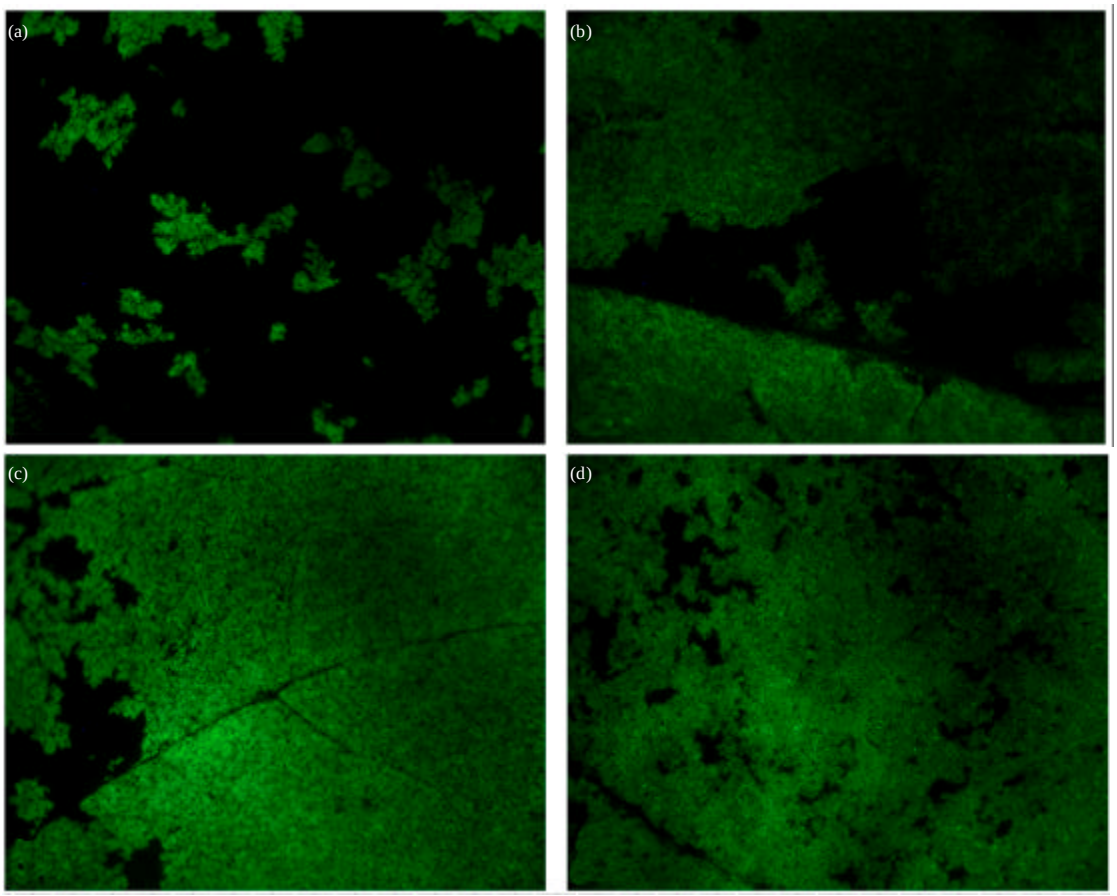

Fig. 3(a-d): Microscopic observation of GFP expression in Ag/pEAQ-HT-GFP-infiltrated plant leaves at 4 Dpi upon receiving vacuum infiltration treatment at (a) 400 mmHg for 2 min, (b) 400 mmHg for 3 min, (c) 600 mmHg for 2 min and (d) 600 mmHg for 3 min. No similar fluorescence signals were seen in negative control Ag/pEAQ-HT-infiltrated plants

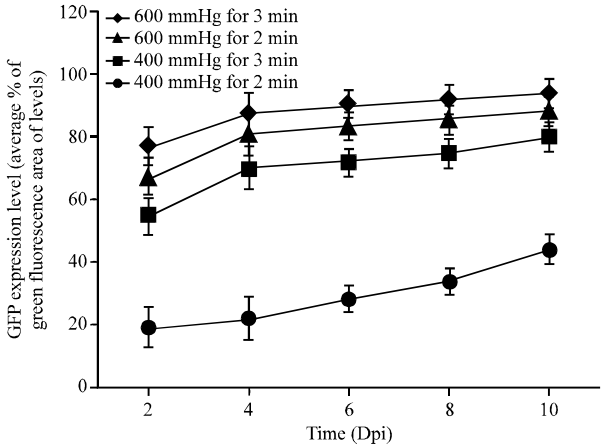

Fig. 4: Representative curves of GFP expression level expressed in percentage of green fluorescence area per total leaf area observed under fluorescence microscopy using a GFP filter at 2, 4, 6, 8 and 10 Dpi. In general, all four vacuum infiltration conditions showed a time-dependent growth on the fluorescence level. The mean GFP expression area percentages ranked in an ascending order as 400 mmHg for 2 min<400 mmHg for 3 min<600 mmHg for 2 min<600 mmHg for 3 min of the different infiltration conditions are statistically significant ($p<0.05$). Error bars show standard error of mean values

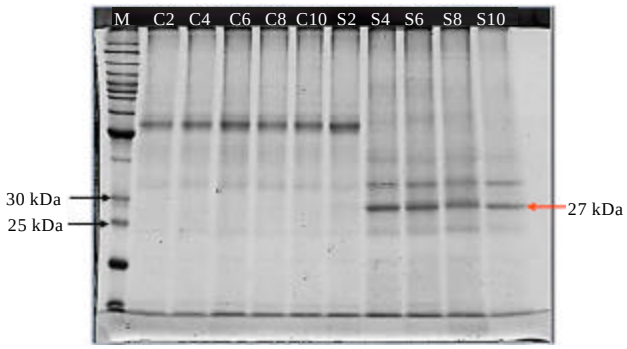

Fig. 5: SDS-PAGE profiles of total soluble proteins extracted from *N. benthamiana* leaves subjected to 400 mmHg for 3 min infiltration condition at 2, 4, 6, 8 and 10 Dpi, GFP bands at a molecular size of 27 kDa are seen (red arrow) in Ag/pEAQ-HT-GFP-infiltrated leaves only but not in the negative control (Ag/pEAQ-HT). Lane M, BenchMark™ pre-stained protein ladder (Invitrogen, USA) with two molecular sizes indicated (black arrows). Lanes C2-C10, extracts from leaves infiltrated with the negative control (Ag/pEAQ-HT). Lanes S2-S10, extracts from leaves infiltrated with Ag/pEAQ-HT-GFP

SDS-PAGE profiles: Total soluble proteins had been successfully extracted from leaf samples of all infiltration conditions tested. One of the representative SDS-PAGE profiles is shown in Fig. 5. It can be observed that the expected protein bands with a molecular size of 27 kDa (similar to GFP) were evidenced in leaf samples receiving vacuum infiltration of Ag/pEAQ-HT-GFP but not in those of the negative control samples (Ag/pEAQ-HT-infiltrated).

Confirmation of GFP expression by Western blotting: The GFP protein bands with a molecular size of 27 kDa were successfully detected in leaf samples of Ag/pEAQ-HT-GFP-infiltrated plants with different infiltration conditions (Fig. 6a-d, Lanes S2-S10) but not in those of Ag/pEAQ-HT-infiltrated plants that served as negative controls (Fig. 6a-d, Lanes C2-C10). This hence further confirmed the GFP expression at molecular level. GFP expression had started as early as 2 Dpi but the level was much lower comparing to later Dpi for all infiltration conditions.

Figure 7 shows the kinetic GFP expression originated from Western blotting represented in reflective density (RD mm^{-2}). There were slight fluctuations on protein density from one time point to another. The infiltration conditions and their GFP band reflective densities obtained at 10 Dpi are summarized as follows: 400 mmHg for 2 min (192.8 RD mm^{-2}), 400 mmHg for 3 min (252.4 RD mm^{-2}), 600 mmHg for 2 min (314.5 RD mm^{-2}) and 600 mmHg for 3 min (455.2 RD mm^{-2}). The maximal expression densities achieved: 400 mmHg for 2 min at 10 Dpi, 400 mmHg for 3 min at 8 Dpi, 600 mmHg for 2 min at 4 Dpi and 600 mmHg for 3 min at 10 Dpi were observed.

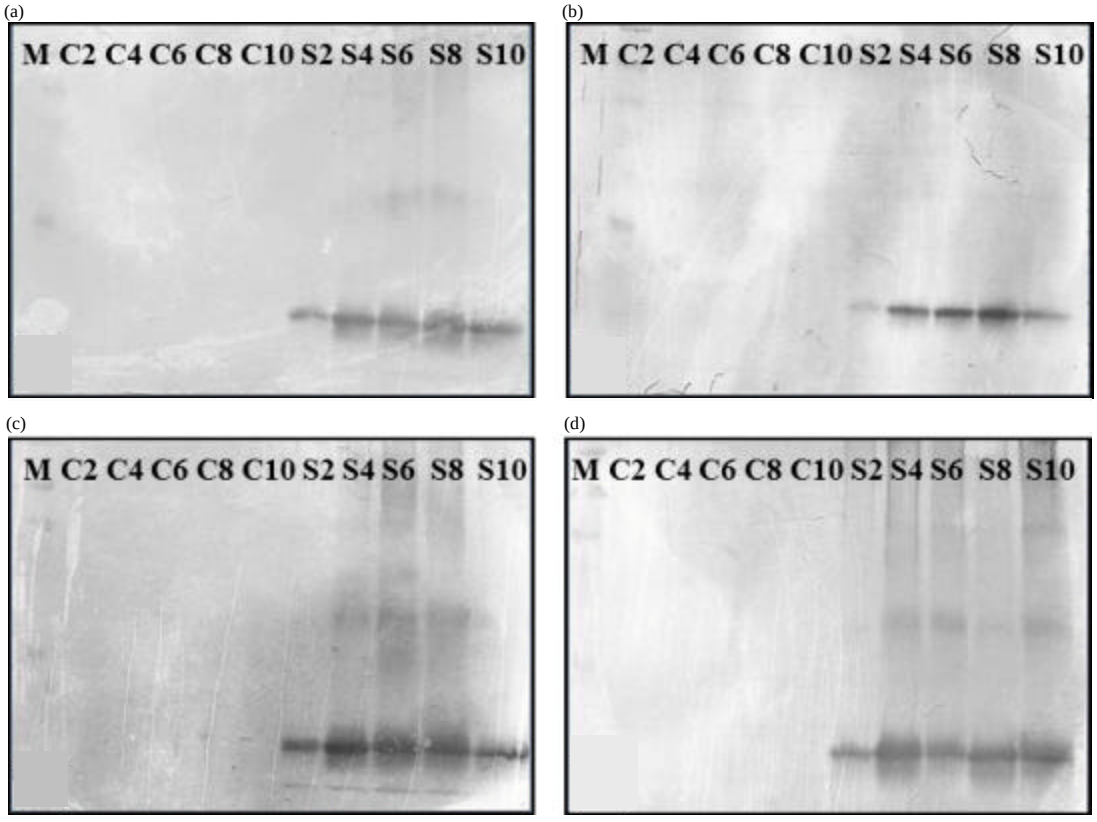

Fig. 6(a-d): Western profiles of total soluble proteins obtained from *N. benthamiana* leaves showing GFP bands at a molecular size of 27 kDa following vacuum infiltration at (a) 400 mmHg for 2 min, (b) 400 mmHg for 3 min, (c) 600 mmHg for 2 min and (d) 600 mmHg for 3 min at 2, 4, 6, 8 and 10 Dpi detected by anti-GFP polyclonal antibody (Abcam, UK). Lane M, Spectra Multicolor broad range protein ladder (Fermentas, USA). Lanes C2-C10, extracts from leaves infiltrated with the negative control (Ag/pEAQ-HT). Lanes S2-S10, extracts from leaves infiltrated with Ag/pEAQ-HT-GFP

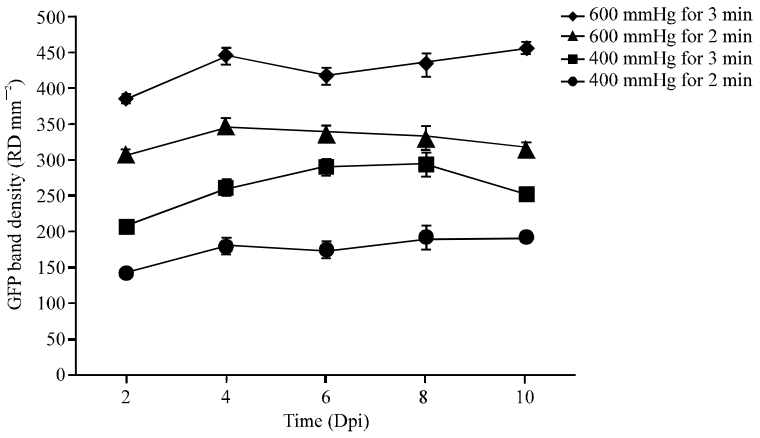

Fig. 7: Reflective density curves generated by GFP protein bands reacted with anti-GFP polyclonal antibody (Abcam, UK) in Western blotting at 2, 4, 6, 8 and 10 Dpi. The maximal expression densities achieved: 400 mmHg for 2 min at 10 Dpi, 400 mmHg for 3 min at 8 Dpi, 600 mmHg for 2 min at 4 Dpi and 600 mmHg for 3 min at 10 Dpi are observed. The mean GFP band densities ranked in an ascending order as 400 mmHg for 2 min<400 mmHg for 3 min<600 mmHg for 2 min<600 mmHg for 3 min of the different infiltration conditions are statistically significant ($p<0.05$). Error bars show standard error of mean values

DISCUSSION

There is a large and fast growing list of plant-made proteins, either pharmaceutical-related or industrial-important, have been expressed by various plant systems. Nowadays, it is more favorable to employ an engineered plant virus-based vector in cloning a gene of interest and express the target protein transiently in plants owing to its speediness and higher yield obtained comparing to stable transformation. Delivery approach of using *Agrobacterium*-mediated infiltration has been proven to be effective. Vacuum agroinfiltration results in homogenous expression of all leaves, making the biomass can be easily scaled up to obtain higher quantity of target protein.

At the first stage, this study entails transformation of *A. tumefaciens* strain LBA4404 with the cowpea mosaic virus (CPMV)-based vectors (pEAQ-HT and pEAQ-HT-GFP) by electroporation and subsequent agroinfiltration of *N. benthamiana* leaves. Agroinfiltration has been proven to be a very suitable method for the delivery of CPMV constructs into plants (Sainsbury *et al*., 2009a). Green Fluorescence Protein (GFP) used in the present study, is an *in vivo* reporter which can facilitate real-time visualization of the temporal patterns of gene expression in *N. benthamiana* following agroinfiltration. First of all, partial optimization of agroinfiltration was performed to investigate the effect of agrobacterial concentration on leaf injury. It is known that low agrobacterial concentration reduces transformation efficiency whereas high agrobacterial concentration damages the plant cells (Ozawa, 2009). Three OD_{600} values, which are 0.3, 0.6 and 1.0 were infiltrated using a syringe without needle into the plant leaves. The use of syringe instead of vacuum is to prevent a confusion of leaf injury which could be due to vacuum infiltration procedure. Physical lesions caused by a syringe at the inoculation sites could be easily identified and therefore are distinguishable from any chlorotic or necrotic symptom that resulted by *A. tumefaciens*. According to the results obtained (Fig. 1a), a high absorbance value (OD_{600} = 1.0) of *A. tumefaciens* had induced severe chlorosis and necrosis on leaves leading to wilting. In fact,

this hypersensitive response of plant leaves to high level of *A. tumefaciens* infection had been reported previously (Kuta and Tripathi, 2005; Pruss *et al.*, 2008). So, OD_{600} = 0.3 where no undesirable chlorotic or necrotic symptom observed is chosen to be the optimum agrobacterial concentration for the vacuum infiltration procedure. Moreover, it was in agreement with several researches that *A. tumefaciens* OD_{600} ranging from 0.2 to 0.5 (Alamillo *et al.*, 2006; Komarova *et al.*, 2006; Pua *et al.*, 2012) are ideally used.

Next, the simple vacuum agroinfiltration system was set up as illustrated in Fig. 2 and used for the subsequent optimization procedure. The general components of the system include vacuum jar with lid (can also be used as vacuum desiccator), vacuum pump with regulator, aluminum foil, 500 mL beaker and two spatulas which can be found in a basic laboratory. The specific elements like *Agrobacterium* suspension, autoclaved distilled water and six week-old *N. benthamiana* plants are required to prepare in advance. The system can be adaptable to fit into individual preference. Likewise, soil in the pot can also be covered with thick plastic sheet or cardboard instead of aluminum foil. The volume of the infiltration solution and beaker can be justified as well. The two spatulas placing inside the beaker is in fact to allow a gap between the pot and beaker to facilitate maximum expulsion of air from the leaf stomata when vacuum pressure is released and this increases delivery effectiveness of agrobacterial suspension into plant tissues.

In the other hand, it is worth to note that this study established a highly cost-effective method for transient expression in which only autoclaved distilled water and *Agrobacterium* were used as components of the infiltration medium contrasting to the vacuum infiltration approaches developed by previous studies (Bechtold and Pelletier, 1998; Ye *et al.*, 1999; Tague and Mantis, 2006; Pua *et al.*, 2012) that used costly acetosyringone and 2-(N-morpholino)ethanesulfonic acid (MES). This idea was adopted from previous study (Knight *et al.*, 2010) employing autoclaved distilled water for *Agrobacterium*-mediated nuclear transformation which is different from our transient expression approach. As this approach was done to mimic the nature of *Agrobacterium* infection process, only negligible osmosis problem was observed. Nevertheless, the feasibility of using autoclaved distilled water as infiltration buffer for vacuum agroinfiltration has been proven. This serves as a cost-effective approach which eliminates the use of costly infiltrated buffer consisting of acetosyringone and MES; therefore it definitely further helps in decreasing the cost for producing recombinant proteins in plants. In fact, exogenous acetosyringone functions as phenolic compound to enhance *Agrobacterium* virulence genes activity, especially for monocots (Rahman *et al.*, 2011). Since a dicot plant, *N. benthamiana* used in this study is capable to synthesize acetosyringone at wounded sites (Stachel *et al.*, 1985), the addition of acetosyringone could therefore be omitted.

Since previous reports showed that pressure and duration of vacuum treatment had significant effect on the vacuum agroinfiltration of *Lactuca sativa* (Simmons *et al.*, 2009; Li *et al.*, 2007). The optimizations were focused on the vacuum pressure and treatment time required for an effective vacuum agroinfiltration. The plant expression of GFP has been determined at functional and molecular levels by fluorescence microscopy and Western blotting, respectively for 10 days upon receiving different infiltration conditions. Basically, GFP expression has been detected throughout the course of study in an increasing manner with the lowest level at 2 Dpi and highest level at 10 Dpi based on fluorescence microscopic assessment (Fig. 3). All four infiltration conditions showed the same tendency (Fig. 4). The absence of GFP signal in the negative control (Ag/pEAQ-HT-infiltrated) leaves has confirmed the specificity of the fluorescence filter used and ruled out the possibility of plant-derived autofluorescence. In addition, GFP bands with a molecular

size of 27 kDa were detected in Ag/pEAQ-HT-GFP-infiltrated plants only by Western blotting analysis reacted with anti-GFP antibody and therefore further confirmed the antigenicity (Fig. 6). Generally, the GFP was expressed at five time points tested (2 to 10 Dpi) but the observation with the steepest increment from 2 to 4 Dpi was conforming to previous findings (Wroblewski *et al.*, 2005; Wydro *et al.*, 2006; Sheludko *et al.*, 2007). Sheludko *et al.* (2007) found that GFP accumulation achieved the maximal level at 3 Dpi and remained stable up to 8 Dpi. Based on these studies, it is believed that the GFP expression can still be detected within a month's period. Nevertheless, this short course of GFP expression study is sufficient to demonstrate the optimal condition in the vacuum agroinfiltration process.

Although, the trend curves of fluorescence areas (Fig. 4) and reflective densities (Fig. 7) are seemed to be slightly different, Pearson's correlation had been conducted to compare relatively these semi-quantitative results obtained from fluorescence microscopy and Western blotting. A correlation coefficient of 0.887 ($p<0.01$) was obtained indicates that the two assays resultant findings have rather good linear relationship that can jointly upshot a common conclusion. When summing up the fluorescence areas (Fig. 4) and reflective densities (Fig. 7) of all time points for each infiltration condition, a rank of expression level in an ascending order can be written as 400 mmHg for 2 min <400 mmHg for 3 min<600 mmHg for 2 min<600 mmHg for 3 min which is applied similarly to both fluorescence microscopy and Western blotting. In brief, 600 mmHg for 3 min had the highest mean values while 400 mmHg for 2 min had the lowest. Both vacuum pressure and time had a significant effect ($p>0.05$) on the level of GFP expression in the leaves of *N. benthamiana*. However, plants subjected to vacuum treatment at 600 mmHg showed chlorotic and necrotic morphology and increasingly became weaker starting from 6 Dpi onwards (data not shown). For instance, shorter recombinant proteins than GFP (27 kDa) that can be expressed abundantly are recommended to use vacuum agroinfiltration at 600 mmHg for 3 min and harvest within 5 Dpi. In contrast, plants infiltrated at 400 mmHg generally looked healthier and stronger throughout the study period. Tague and Mantis (2006) reported that vacuum agroinfiltration of *Arabidopsis thaliana* at 400 mmHg for 3 min was highly effective. Therefore, vacuum agroinfiltration at 400 mmHg for 3 min can be employed in the production of recombinant proteins that are larger in molecular size and require longer period to optimally express and assemble into their biologically active form such as plantibodies.

Yet, there are a few limitations of study have been realized. These include unrevealed actual amount of GFP expression per milligram of infiltrated leaf tissues and narrow range of parameters tested, by which the minimum and maximum pressure and duration for vacuum infiltration have not been determined. Nevertheless, an in-house laboratory-scale vacuum agroinfiltration system has been developed and is proven to be practical to express a recombinant protein using a cheaper infiltration solution. Moreover, based on the infiltration optimization findings, it has been suggested to use different infiltration treatments for producing various heterologous proteins depending on protein nature and plant response as above discussed.

CONCLUSION

The speedy and high expression capacities of plant viral vector; effective and systemic delivery of *Agrobacterium* and low production cost of plants have been apprehended as the advantages of vacuum agroinfiltration. The present study has illustrated a simple, practical and cost-effective laboratory-scale vacuum-assisted agroinfiltration system. It can be used in a basic laboratory to

conduct plant molecular pharming researches which potentially lead to the production of high value products at lower cost in future. Till then, more people in need particularly those from developing countries would definitely be benefited.

ACKNOWLEDGMENTS

We thank Professor George Lomonossoff (John Innes Centre, Norwich, United Kingdom) for supplying the plant viral vectors in kind. This study was conducted in Biotechnology Research Centre, University of Nottingham Malaysia Campus.

REFERENCES

Alamillo, J.M., W. Monger, I. Sola, B. Garcia and Y. Perrin *et al.*, 2006. Use of virus vectors for the expression in plants of active full-length and single chain anti-coronavirus antibodies. Biotechnol. J., 1: 1103-1111.

Bechtold, N. and G. Pelletier, 1998. *In Planta Agrobacterium*-Mediated Transformation of Adult *Arabidopsis thaliana* Plants by Vacuum Infiltration. In: *Arabidopsis* Protocols, Martinez-Zapater, J.M. and J. Salinas (Eds.). Springer, New York, USA., ISBN-13: 9780896033917, pp: 259-266.

D'Aoust, M.A., P.O. Lavoie, J. Belles-Isles, N. Bechtold, M. Martel and L.P. Vezina, 2009. Transient Expression of Antibodies in Plants using Syringe Agroinfiltration. In: Recombinant Proteins from Plants: Methods and Protocols, Faye, L. and V. Gomord (Eds.). Springer, New York, USA., ISBN-13: 9781588299789, pp: 41-50.

Gleba, Y., V. Klimyuk and S. Marillonnet, 2005. Magnifection: A new platform for expressing recombinant vaccines in plants. Vaccines, 23: 2042-2048.

Knight, C.J., A.M. Bailey and G.D. Foster, 2010. Investigating *Agrobacterium*-mediated transformation of *Verticillium albo-atrum* on plant surfaces. PLoS ONE, Vol. 5. 10.1371/journal.pone.0013684

Komarova, T.V., M.V. Skulachev, A.S. Zvereva, A.M. Schwartz, Y.L. Dorokhov and J.G. Atabekov, 2006. New viral vector for efficient production of target proteins in plants. Biochemistry, 71: 846-850.

Kuta, D.D. and L. Tripathi, 2005. *Agrobacterium*-induced hypersensitive necrotic reaction in plant cells: A resistance response against *Agrobacterium*-mediated DNA transfer. Afr. J. Biotechnol., 4: 752-757.

Li, J., M. Chen, X.W. Liu, H.C. Zhang, F.F. Shen and G.P. Wang, 2007. Transient expression of an active human interferon-beta in lettuce. Scientia Horticulturae, 112: 258-265.

Ma, J.K.C., P.M.W. Drake and P. Christou, 2003. The production of recombinant pharmaceutical proteins in plants. Nat. Rev. Genet., 4: 794-805.

Menassa, R., V. Nguyen, A. Jevnikar and J. Brandle, 2001. A self-contained system for the field production of plant recombinant interleukin-10. Mol. Breed., 8: 177-185.

Natorajan, D., H.Y. Yong, I. Ismanizan and Z. Zamri, 2010. Feasibility analysis of leaf disc samples produced via agroinfiltration for promoter trapping studies. Emirates J. Food Agric., 22: 448-455.

Ozawa, K., 2009. Establishment of a high efficiency *Agrobacterium*-mediated transformation system of rice (*Oryza sativa* L.). Plant Sci., 176: 522-527.

Pruss, G.J., E.W. Nester and V. Vance, 2008. Infiltration with *Agrobacterium tumefaciens* induces host defense and development-dependent responses in the infiltrated zone. Mol. Plant-Microbe Interact., 21: 1528-1538.

Pua, T.L., H.S. Loh, F. Massawe, C.S. Tan and A.R. Omar, 2012. Expression of insoluble influenza neuraminidase type 1 (NA1) protein in tobacco. J. Trop. Life Sci., 2: 62-71.

Rahman, Z.A., Z.A. Seman, N. Basirun, A.L. Julkifle, Z. Zainal and S. Subramaniam, 2011. Preliminary investigations of *Agrobacterium*-mediated transformation in indica rice MR219 embryogenic callus using *gusA* gene. Afr. J. Biotechnol., 10: 7805-7813.

Sainsbury, F. and G.P. Lomonossoff, 2008. Extremely high-level and rapid transient protein production in plants without the use of viral replication. Plant Physiol., 148: 1212-1218.

Sainsbury, F., E.C. Thuenemann and G.P. Lomonossoff, 2009a. pEAQ: Versatile expression vectors for easy and quick transient expression of heterologous proteins in plants. Plant Biotechnol. J., 7: 682-693.

Sainsbury, F., L. Liu and G.P. Lomonossoff, 2009b. Cowpea Mosaic Virus-Based Systems for the Expression of Antigens and Antibodies in Plants. In: Recombinant Proteins from Plants: Methods and Protocols, Faye, L. and V. Gomord (Eds.). Springer, New York, USA., ISBN-13: 9781588299789, pp: 25-39.

Sainsbury, F., M.C. Canizares and G.P. Lomonossoff, 2010. *Cowpea mosaic* Virus: The plant virus-based biotechnology workhorse. Annu. Rev. Phytopathol., 48: 437-455.

Sheludko, Y.V., Y.R. Sindarovska, I.M. Gerasymenko, M.A. Bannikova and N.V. Kuchuk, 2007. Comparison of several *Nicotiana* species as hosts for high-scale *Agrobacterium*-mediated transient expression. Biotechnol. Bioeng., 96: 608-614.

Simmons, C.W., J.S. VanderGheynst and S.K. Upadhyaya, 2009. A model of *Agrobacterium tumefaciens* vacuum infiltration into harvested leaf tissue and subsequent in planta transgene transient expression. Biotechnol. Bioeng., 102: 965-970.

Stachel, S.E., E. Messens, M. Van Montagu and P. Zambryski, 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature, 318: 624-629.

Tague, B.W. and J. Mantis, 2006. In Planta Agrobacterium-Mediated Transformation by Vacuum Infiltration. In: *Arabidopsis* Protocols, Salinas, J. and J.J. Sanchez-Serrano (Eds.). Springer, New York, USA., ISBN-13: 9781588293954, pp: 215-223.

Tremblay, R., D. Wang, A.M. Jevnikar and S. Ma, 2010. Tobacco, a highly efficient green bioreactor for production of therapeutic proteins. Biotechnol. Adv., 28: 214-221.

Wroblewski, T., A. Tomczak and R. Michelmore, 2005. Optimization of *Agrobacterium*-mediated transient assays of gene expression in lettuce, tomato and *Arabidopsis*. Plant Biotechnol. J., 3: 259-273.

Wydro, M., E. Kozubek and P. Lehmann, 2006. Optimization of transient *Agrobacterium*-mediated gene expression system in leaves of *Nicotiana benthamiana*. Acta Biochimica Polonica, 53: 289-298.

Ye, G.N., D. Stone, S.Z. Pang, W. Greely, K. Gonzalez and M. Hinchee, 1999. *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. Plant J., 19: 249-257.

Yusibov, V. and T. Mamedov, 2010. Plants as an alternative system for expression of vaccine antigens. Proc. ANAS (Biol. Sci.), 65: 195-200.