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Regeneration and Transformation of Gene Encoding the Hemagglutinin Antigen of the H5N1 Virus in Frond of Duckweed (*Spirodela polyrhiza* L.)

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Abstract

H5N1 (Avian influenza virus) has caused economic loss and remained a serious human health risk in many countries, including Vietnam. Plant-derived vaccines have offered the advantages over tradition subunit vaccine with low-cost production potential. Duckweed (*Sprirodela polyrhiza* L.) has found a valuable target plant for various application. In this study, we have attempted to re-generate and transform the gene encoding the hemagglutinin antigen of H5N1 virus in frond of *S. polyrhiza*, a widely great duckweed grown in Vietnam. The results have shown that the culture medium for *S. polyrhiza* duckweed is Hutner medium with reduced mineral concentration ½ (H/2) supplemented with 10g/l sucrose at pH 6. This medium showed appropriate for the best growth and development of duckweed. Results of PCR analysis of *S. polyrhiza* lines from 14 transformation experiments that has obtained 4 duckweed lines carrying *HA1* gene. This prelinary results will be provide the basis for transgenic research in the dukweed species and be useful for further generate the producing enable vaccine via the transgenic plants

Keywords: Duckweed**,** H5N1, Vaccine, Transgenic plant

1. Introduction

H5N1 (Avian influenza virus) was first believed to cause disease only in poultry until the initial outbreak of highly pathogenic in humans in Asia in 1997. It has caused economic loss and

remained a serious human health risk in many Asian countries, including China, Vietnam, Thailand, Korea, Indonesia, Japan etc. Hemagglutinin (HA), the principal viral surface antigen, is the primary target for neutralizing antibodies and is responsible for binding of the virus to host receptors, enabling entry into the host cell through endocytosis and subsequent membrane fusion (Stevens *et al*., 2006; Guo *et al*., 2012). In the trend of recent advanced biotechnology research have exhibited some achievements, including system for the production of desired vaccine antigen in transgenic plants (Floss *et al*., 2007). Plant-derived vaccines offer advantages over tradition subunit vaccine with low-cost production potential (Franconi *et al*., 2010). The duckweed (*Lemnaceae*) is a monocotyledonous family of five genera: *Spirodela, Lemna, Landoltia, Wolffia* and *Wolfiella*, and included over 37 species. Among them, *Spirodela* is the most ancestral, while *Wolffia* is the most derived (Les *et al*., 2002). All *Lemnaceae* species are small aquatic plants, free-floating, beneath or totally cover the furface of fresh water and wetlands, often forming dense, homogeneous clonal populations (Stomp, 2005). They proliferate primarily through vegetative budding of new fronds from parent fronds. Newly budded fronds remain attached to the parent frond to varying degrees, depending on species. Duckweeds have a worldwide distribution, especially temperate and tropical regions. The *Lemna gibba* L. and the *Lemna minor* L. are the most studied species of *Lemnaceae* family in phytoremediation and ecotoxicology (Mkandawire and Dudel, 2005).

Great duckweed (*Spirodela polyrhiza* L.) is belonging to the member of *Lemnaceae* family under the group monocotyledons. The individual plants range in size from 1.5 cm long and is a successive reduction of morphological structures in parallel with evolutionary advancement within the family (Wang *et al*., 2011). Great duckweed is one of important model system for research on plant biology, biofuel potential candidates, biomass utility, toxicity testing organism, biotech protein factories, wastewater remediation, high protein animal feeds, carbon cycling because of its fast growth, wide distribution, short life span and stability to environmental change (Stomp, 2005; Brain and Solomon, 2007; Ozengin and Elmaci, 2007; Rahman *et al*., 2007), particularly in biochemical studies *S. polyrhiza* is an ideal system for biofueld, bioremediation and carbon cycling due to their aspect of fast-growing direct contact with media and has the smallest genome size (~150 Mb) (Wang *et al*., 2011; Wang *et al*., 2014). This aquatic plant consists of high mutrient uptakes rates, and is cold tolerant and less sensitive than other aquatic plants to high nutrient stress, droughts, pests and disease. It develops and grows well in water under the different climates at temperature between 6 to 33^0C, especially in tropical and temperate areas and obtains high biomass yield approximately 10-30 tons DM/ha/year, containing high levels of crude protein up to 10 tons/ha/year with a good amino acid balance (Leng *et al*., 1995).

Vietnam is a tropical and subtropical country where the duckweeds, especially, *S. polyrhiza* is widely speading and growing in the streams, ponds and wetland areas. Its production plays a key role in this country, provides meat and eggs in the diet of people and income from local sale in domestic markets. Traditional vaccines are produced by applying fermentation technology in various cell culture systems. However, numerous limitation related to fermentation so that development of alternative systems for the production of vaccines is timely (Davoodi *et al*., 2009; Daniell *et al*., 2009; Gomez *et al*., 2010).

Since duckweed is found a valuable target plant for various application, the feasibility of transformation of *S. polyrhiza* by *Agrobacterium tumefaciens*- mediated gene transfer may offer an extended range of application. In this study, we have attempted to re-generate and transform the gene encoding the hemagglutinin antigen of H5N1 virus in frond of *S. polyrhiza*, a widely great duckweed grown in Vietnam. The data should be useful for further generate the producing enable vaccine via the transgenic plant.

2. Materials and Methods

2.1 Plant Materials

Duckweeds were collected in the experimantal field of Agricultural Genetics Institute, Hanoi, Vietnam, and then cultured in the jar at nursery of Agricultural Genetics Institute. The samples were identified and confirmed as the *S. polyrhiza* by the method of Landolt (1986)

2.2 Genetic Materials

A. tumefaciens AGL-1 carrying p6d35S-UbiHA1 vector which containing selectable marker gene *hpt* (resistant hygromycin) and gene *HA1* encoding the hemagglutinin antigen of the H5N1 virus is under the control of the Ubiquitin promoter was used.

2.3 In Vitro Starting Materials

To make *in vitro* starting materials, $Ca(OCl)_2$ with diffirent concentrations at 3, 5 and 7%, and with time duration was s 2,4 and 6 min to sterile the plants *S. polyrhiza*. After soaking with antiseptic chemmical, duckweed samples were rinsed many times by sterile water, then sucked dried and cultured on solid H/5 added 15g/l sucrose at pH 5.8. Each formula was used 100 cluster *S. polyrhiza* including 3 – 4 fronds/ cluster.

2.4 Optimization of Culture Medium for Duckweed in In Vitro

The basic medium for duckweed culture was conducted following the method of Hutner (1975) with some modifications. Briefly, the optimal culture medium for *S. polyrhiza* was prepared: Medium H was added 15g/l sucrose, pH 5.8 with mineral content at ratios: 1/1;1/2; 1/3;1/4 and 1/5 (H; H/2; H/3; H/4 and H/5). Medium H/2 (50% mineral Hutner) was added 15g/l sucrose with variable medium pH at 4.0; 5.0; 6.0; 7.0; 8.0; 9.0 and 10, respectively. Medium H/2 with sucrose concentrations was added (0, 10, 20 and 30 g/l). Duckweed was cultured in RUMED incubator, temperature at 26 $\pm 2^{o}$C, humidity 80%, photoperiod 10/24h.

2.5 Method for Gene Transfer in Spirodela Polyrrhiza Mediated Agrobacterium

The transformation protocol was performed by the method of Boehm *et al* (2001), with some minor modifications (Thu *et al*., 2010).

2.5.1 Preparation of Agrobacterium Suspension

Streak *Agrobacterium* from -20 ℃ stock (in glycerol) on solid LB medium with the appropriate antibiotics (kanamycin 50 mg/l and carbenicillin 50 mg/l).The cultured growth was kept for 3 days at 28 ℃ in darkness. Pick up single colonies and streak it on liquid H/2 medium (pH 5.6) added 200 µM AS, shaking culture at 160 rpm for 4-6 h. Before inoculation with duckweed, the

density of bacteria reached $OD_{600} \approx 0.5$-1.0 was adjusted.

2.5.2 Preparation of Duckweed Sample

Three days prior to the transformation experiment, the duckweed should be cultured in the appropriate medium to assure the homogeneous of target materials. The culture medium of *S. polyrhiza* was H/2 + 10 g/l sucrose + 7g/l agar at pH 6.0

2.5.3 Infection and Cocultivation

Infection stage was carried out in the effendorf tube including duckweed fronds with 1 ml bacteria suspension. This mixture was centrifuged and vacuumed in 10 min, then kept at room temperature for 20 min. Cocutivation time between bacteria and duckweed sample was 3 days at 26^0C, humidity 85%, light 3000 lux with photoperiod 12/24 h.

2.5.6 Removing *Agrobacterium* and Resting Culture

After cocultivation, transfer the duckweed to resting medium which was growth medium of duckweed adding 200 mg/l timentin at pH 6. Subcultured on this medium from 2-3 cycles

2.5.7 Selection of Transformed Duckweed

After removing *Agrobacterium* and resting culture, the transformed duckweed frond to selection medium (Medium containing selective agent was hygromycin) was transfered. Maintain and subcultured transformed duckweed lines on selection medium from 3-5 cycles (5-7 days/cycle) were conducted with the selected medium was H/2 + 5 mg/l hygromycin + 7g/l agar

2.6 PCR Analysis of Transgenic Duckweed Lines

The PCR was performed with the specific primers for *hpt* and *HA1* genes

Primer sequences:

Hpt- F- 5'- AGAAGAAGATGTTGGCGACCT-3'

Hpt- R- 5'- GTCCTGCGGGTAAATAGCTG-3'

T-*HA1*-for- 5'TACCCAGGGGATTTCAATGAC 3'

T-*HA1*-rev - 5'GACACTTGGTGTTGCAGTTAC 3'

2.7 Evaluation of Factors

Each experimental formula using 5 triangle vases contained 100 ml medium with 20 cultured duckweed fronds. Counting duckweed frond total in 5 vases of every experimental formula after culture 24 h. Experimental time was 6 days. Each experimental formula was repeated 3 times.

Frond multiplying rate (Landolt & Kandeler, 1987)

$$k_{ni} = \frac{Fd_i}{Fd_0}$$

(Of which k_{ni}: Multiplication factor at the i day; Fd_i: Total number of leaflike at i culture day; Fd_0: total leaflike in initial time)

3. Results and discussion

3.1 Making in Vitro Starting Materials

As shown in Table 1, the concentration and time duration showed significant influence on the ration of the steriled samples. The highest ration of uncontaminated sterile sample was 54% when applying decontaminated method Ca $(OCl)_2$ 5% in 2 min. After sterilization, a part of around nodule point of duckweed frond died partially but 2 – 3 late days appearing new duckweed frond growth from its meristem. The sterile method by applying Ca $(OCl)_2$ 5% for 2 min to make *in vitro* starting materials for further studies.

Table 1. The effect of time and concentration Ca $(OCl)_2$ to the ratio of steriled samples of *S. polyrhiza*

Ca (OCl)2 (%)	Time (minute)	The ratio of steriled sample (%)	The ratio of contaminated sample (%)	The ratio of death sample (%)
3	2	28	55	17
	4	32	44	24
	6	46	29	25
5	2	54	24	22
	4	41	20	39
	6	35	17	48
7	2	32	20	48
	4	22	14	64
	6	15	11	74

3.2 Optimization of Culture Medium for Duckweed in Vitro

To produce the starting materials for transformation which must be make optimal conditions for the growth and development of plant. The purpose of this experiment to determine the norm for the growth and development of duckweed as the reproductive characteristic, duplicate time and biomass multiplication factor in duckweed

3.2.1 Effect of Hutner Mineral Content to the Growth and Development of *S. polyrhiza*

Hutner mineral has been by many authors to multiply species belonging to Lemnaceae familia, especially Hutner medium with mineral component decrease 5 times (Landolt, 1986). Therefore, the aims of this experiment to find out the proportion of mineral Hutner suitable for the growth and development of *S. polyrhiza*. In order to select the appropriate mineral content for the growth and development of *S. polyrhiza*, the different mineral ratios: H (100% Hutner mineral), H/2, H/3, H/4, H/5 were applied, the environmental disinfection before being

adjusted to pH 5.8. The result was collected after 24 h culture in 6 days as shown in the Table 2.

Table 2. Effect of Hutner mineral content to the frond multiplying rate in *S. polyrhiza*

Cultute time (days) / H mineral content	0	1	2	3	4	5	6
H	1	1.41	1.89	2.82	3.63	4.23	4.78
H/2	1	1.40	1.83	2.53	3.89	4.87	5.32
H/3	1	1.40	1.74	2.27	3.28	3.96	4.55
H/4	1	1.38	1.69	2.14	3.02	3.50	4.40
H/5	1	1.38	1.56	2.23	2.58	3.34	4.23

The results of Table 2 revealed that: In the first 3 days of culture, there was no significant difference between the formulas of experimental medium, but from fourth, there was significant difference between the medium formulations containing with different Hutner minerals content. The growth of duckweed *S. polyrhiza* was directly proportional with the rate of mineral in the culture medium. However, the growth and development of *S. polyrhiza* on the H medium (100% Hutner mineral) less than H/2 (50 % Hutner mineral) by overlaying fronds, which has not separated, affected to the growth and development of mother fronds, leading to deformed fronds, distortion.

After 6 days of culture, medium formula H/2 (50 % Hutner) for the speed of highest frond multiplying coefficient, reaching 5.32 times, duckweed growth and good development. Healthy duckweed frond, duckweed status similar duckweed living in a natural medium. Therefore, the mineral H/2 background for the next growing experiments was selected.

3.2.2 Effect of pH Medium to the Growth and Development of *S. Polyrhiza*

As the previous report of Landolt & Kandeler (1987), *S. polyrhiza* could grow in medium with a pH from 3.7 to greater than 9 and grows well in the pH range from 4-7.With the aim of finding the most suitable pH for growth and development of *S. polyrhiza in vitro* culture conditions, we have conducted 7 experimental formula on mineral environment with H / 2 + 15 g / l sucrose with a pH instead ranging from 4-10. The obtained results indicated that pH ranging from 5.0 to 9.0 is appropriate for the growth and development of *S. polyrhiza* in laboratory conditions. While, pH lower 5.0 exhibited growth inhibition, aging duckweed and death very quickly. The appropriate pH showed remarkerble enhancement frond multiplying coefficient after 5-6 days of culture. The current data demonstrated that *S. polyrhiza* can grow and develop best at pH = 6, frond multiplier factor was 6.11 times after 6 days of culture, the morphological of duckweed frond *in vitro* was similar as ducweed growth in the nature condition. On the medium having pH > 6, thin duckweed frond, the wide and tend to lean towards the yellow. Therefore, culture medium with pH = 6 for our experiments in the next phase of growth was applied. In the report of Caiedo *et al*. (2000), the inhibition of duckweed growth by ammonium was found to be due to a combined effect of ammonium ions and depending on the pH. In the previous research, Nguyen *et al* (2012) reported to successfully express an avian influenza HA protein in the *Lemna* auxotroph platform.

Table 3. Effect of pH medium to the frond multiplying rate in *S. polyrhiza*

Cultute time (days) / pH	0	1	2	3	4	5	6
4	1	1.36	1.67	2.23	3.26	3.47	3.94
5	1	1.55	1.92	2.64	4.10	4.94	5.20
6	1	1.42	2.03	2.97	4.35	5.34	6.11
7	1	1.50	2.25	3.37	4.38	5.22	5.79
8	1	1.49	2.22	3.33	4.30	5.10	5.61
9	1	1.48	2.32	3.46	4.43	5.01	5.41
10	1	1.50	2.15	3.20	4.23	4.79	5.11

3.2.3 Effect of Sucrose Content to the Growth and Development of *S. Polyrhiza*

Duckweed in general and in particular *S. polyrhiza* is capable of being autotrophic carbon so in medium have not sucrose, it is still able to grow and develop. Howerver, to create material source for experiments, nutrious medium was needed to provide sucrose so that duckweed could grow and develop at rapid speed, providing materials for buiding experiments about generation and transgenic systems and biomass production necessary for the purpose of producing large quantities.

The results have shown that there was of different levels in the tracking target about multiplying coefficient between sucrose supplement formulations and sucrose non-supplement formulations. This difference was greater when the longer incubation time, after 6 days of culture, the multiplier factor in the formula supplemented with 10 g/l sucrose was 1.5 times higher to compare with non-supplemented formula sucrose (Table 4). Supplement formula 10 g/l sucrose for speed and highest multiplying coefficient in culture process, reaching 6.31 times after 6 days of culture.

Table 4. Effect of sucrose content to the frond multiplying rate in *S. polyrrhiza*

Cultute time (days) / Sucrose (g/l)	0	1	2	3	4	5	6
0	1	1.40	1.77	2.95	3.19	3.94	4.22
10	1	1.43	2.31	3.35	4.65	5.55	6.31
20	1	1.46	2.09	3.10	4.35	5.22	6.01
30	1	1.42	1.92	2.91	4.01	4.94	5.65

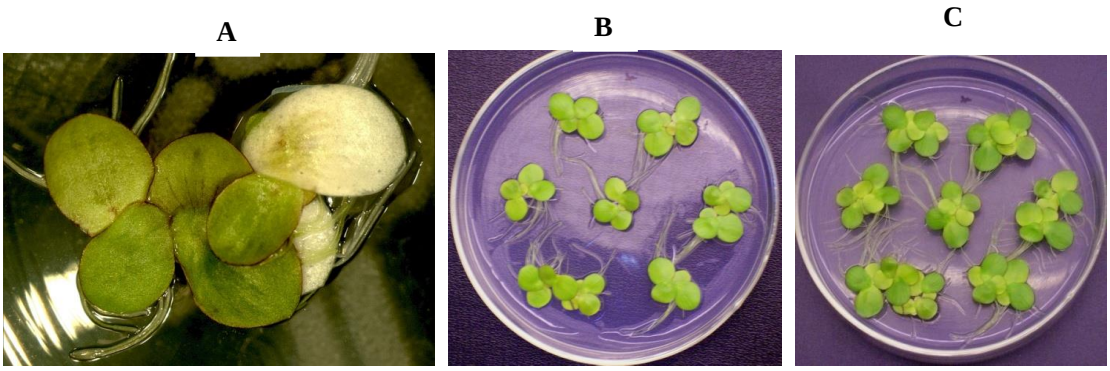

Figure 1. Fronds of *S. polyrhiza* after sterilization (A); new fronds were developed (B); growing on the medium H/2 + 10 g/l sucrose, pH 6.0 (C).

In the other hand, culture medium for *S. polyrhiza* has reached the highest multiplying rate (6.31 times after 6 days of culture) which was H/2 medium additional 10 g / l sucrose at pH 6.0.

3.3 Transformation of Gene Coding to Antigene of H5N1 Virus into S. Polyrhiza Mediated Agrobacterium Tumefaciens

One of the factors that has stimulated the process of transferring T-DNA from *A.tumefaciens* bacteria into plant cells that damaged plant cells. When cells are hurt, it often secretes the phenolic compounds, these compounds have effected to attract bacteria. On the other way, the reproduction of ducweed is vegetative reproduction in the form daughter fronds are generated from the meristem of mother frond. Therefore, we conducted a sample pre-treatment before transformation to improve the efficiency of the gene transfer process.

S. polyrhiza sample was cultured on H/2 medium in 3 days to ensure equivalent – grade of experimental material. Then duckweed fronds was separated from the duckweed cluster and treated according to the method of vertical cut -off, cut through nodes, divided into 2 equal parts frond. Processing sample was then infected with 1 ml of bacterial strains AGL-1 carrying *A. tumefaciens* p6d35S vector of centrifugal vacuum conditions (75 cmHg, 1200 rpm), in 20 min. After infection, duckweed fronds picked out and cultured on co-cultured medium for 3 days. We have conducted a series of *HA1* antigen gene transferring experiments in transgenic duckweed SP. The results are presented in the Table 5. Also, some previous reports showed a reliable transformation protocol of member of the duckweed (*Wolffia Columbiana*) was tested to enhance the accessibility of the plant cells for the infecting A. *tumefaciens* strain LBA4404, harboring p35SGUSINT (Boehm *et al*, 2001). Recently, seedlings of *Lotus corniculatus* were successfully transformed with a gene encoding the H5N1 hemagglutinin (HA) using *Agrobacterium*-mediated transformation (Guo *et al*., 2012).

We obtained 10 transformed duckweed lines after selection from total 14 transformation experiments. This transformed duckweed lines have been multiplied rapidly to generate enough biomass for DNA extraction and analysis of the presence of transgen in the genome of duckweed.

Table 5. Summary of transformation experiments into *S. polyrhiza*

No. of exper.	No of tubes were infected	No of plate was subcultured after selection				No of transformed plants survival after selection
		2 times	3 times	4 times	5 times	
1.	2	0	0			0
2.	3	0	0			0
3.	2	10	0			0
4.	2	18	17	11	3	3
5.	3	10	0			0
6.	2	0				0
7.	2	0				0
8.	3	17	26	20	3	3
9.	2	8	2	0		0
10.	2	10	3	0		0
11.	2	0				0
12.	2	0				0
13.	3	16	13	13	2	2
14.	2	13	7	0	0	0

3.4 PCR Analysis of Transformed Duckweed Lines

3.4.1 Experiment of Hpt Gene

We have conducted PCR analysis using 10 genomic DNA samples of transformed duckweed as a template and specific primers for amplication of *hpt* gene with the size of 714 bp. The electrophoresis results of PCR products have shown in Figure 2 revealed that: Among 10 DNA samples of transformed plants analyzed have 8 samples appeared DNA band at the position having size 714 bp. Therefore, we obtained 8 duckweed lines presence *hpt* gene from 10 lines of transformed duckweed after many selection cycles.

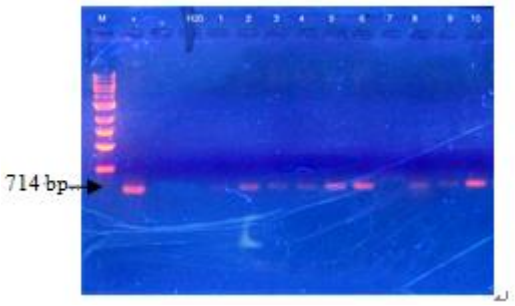

Figure. 2. PCR analysis of transformed duckweed lines for hpt gene

M: Ladder 1kb (Fermentas); (+): DNA plasmid p6d35S-Ubi*HA1*; (-): *S. polyrrhiza* WT (non-transformed); H20; Line 1; 2; 3; 4; 5; 6; 8; 9; 10; 11 transformed lines having presence of *hpt* gene; Line 7: transformed line having absence of *hpt* gene

3.4.2 Experiment on HA1 gene

We conducted PCR analysis using DNA samples of 8 duckweed lines and specific – primers for amplification HA1 gene

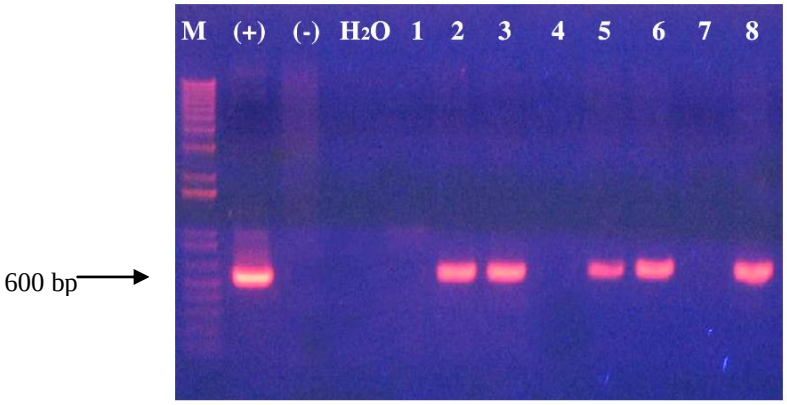

Figure. 3. PCR analysis of transformed duckweed lines for HA1 gene

M: Ladder 1kb (Fermentas); (+1), (+2): DNA plasmid p6d35S-Ubi*HA1*; (-): *S. polyrrhiza* WT (non-transformed); H20; Line 1; 3; 7; 8: transformed lines having presence of *HA1* gene; Line 4, 5, 9: transformed line having absence of *HA1* gene.

The electrophoresis results of PCR products have shown in Figure 3: Among 8 lines of transformed duckweed having positive results with *hpt* gene had 4 lines to appear DNA band at the position ~ 600 bp in size. Thus, we have obtained 4 lines with presence of *HA1* gene from 08 lines of transformed duckweed as templates, these lines are capable of growth and good biomass production which was similar with natural duckweed.

4. Conclusions

The culture medium for *S. polyrhiza* duckweed is Hutner medium with reduced mineral concentration ½ (H/2) supplemented with 10g/l sucrose at pH 6. This medium showed appropriate for the best growth and development of duckweed. Results of PCR analysis of *S. polyrhiza* lines from 14 transformation experiments that has obtained 4 duckweed lines carrying *HA1* gene. This prelinary results will be provide the basis for transgenic research in the dukweed species and be useful for further generate the producing enable vaccine via the transgenic plants.

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