EFFECTS OF PLASMA ACTIVATED WATER (PAW) ON EXPRESSION OF SOME GROWTH RELATED GENES IN NGOC LINH GINSENG (*Panax vietnamensis* Ha et Grushv.) HAIRY ROOTS

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SUMMAY

Previous research confirms that plasma activated water (PAW) has not only bactericidal and anti-cancer properties, but also stimulates seed germination and increases growth of the tissue and cell culture, especially increasing fresh biomass of *in vitro* Ngoc Linh ginseng hairy root culture. In this study, we focused on the study of the impact of PAW on the expression of several genes involved in the growth of Ngoc Linh ginseng hairy roots. The method used was RT-PCR, the selected gene object included *rolB* gene, *GA20ox* gene and *Actin* gene. The obtained results showed that: (1) DNA and RNA can be extracted with good quantity and quality from *in vitro* Ngoc Linh ginseng root tissue culture for further use. (2) Using RNA samples and specific primers for *Actin*, *rolB* and *GA20ox* genes, cDNAs can be synthesized for conducting RT-PCR of the above genes. (3) By adding PAW to the final concentrations from 0 to 20% and the follow-up time after addition of PAW to the culture medium is 12 to 72 hours, it can be shown that *Actin* gene expression is stable; *rolB* gene expression increased after 12 hours of treatment with 5% PAW, but decreased at higher concentrations and after that; *GA20ox* gene alone did not active in Ngoc Linh ginseng hairy roots. From that result, it can be concluded that PAW affects very early and only at low concentration (5%) activates the cell proliferation gene *rolB* in Ngoc Linh ginseng hairy root tissue. Research on the effects of new PAW's with higher content of the active radicals on growth and gene expression is underway to investigate the cell and molecular biology effects of PAW.

Keywords: Plasma activated water; Ngoc Linh ginneng hairy root; actin gene, *GA20ox*, rolB expression.

INTRODUCTION

Plasma technology is being applied in agriculture, medicine, environment for various purposes (Kaushik, 2018). Activation of water by treatment with non-thermal plasma (PAW) (Goossens, 2012) results increases of the concentration of H_2O_2, HNO_2, and HNO_3 proportionaly to activation time from 45 seconds (= 1X) to 900 seconds (= 20X) and the adding of PAW to the media for cultivation of Ngoc Linh hairy roots stimulates growth of fresh biomass (Le Tran Binh *et al*., 2019).

The effect of plasma activating medium on the expression of p53 gene family in cancer cells has been published (Shi *et al*., 2017). Therefore, we focus our research on Ngoc Linh ginseng hairy root cells in order to gain new results to contribute to the understanding of this endemic object of our medicinal plants.

The focus of the research content is on the genes related to the growth of cultivated Ngoc Linh ginseng hairy roots. The previous study of the research group initially found that the environment to add PAW to the liquid culture of Ngoc Linh ginsengroot culture has the effect to increase the fresh weight by 19%. However, we still have not determined what is the mechanism leading to that result. Two assumptions have been made. One is that the plasma-activated water contains free radicals such as nitrite, nitrate, peroxide, and their nitrite and nitrate components can be stable for long period. Therefore, the addition of PAW water may provide some of the extra quantities needed for root development. The second hypothesis is that PAW can influence the expression of key genes that cause that difference. To provide additional evidence to help elucidate assumptions, we conducted a study "Investigating the activity of genes involved in proliferation in Ngoc Linh ginsenghairy root tissues" to identify those that are important. For gene expression testing through reverse transcriptase PCR (RT PCR) to find the relationship between gene activity and the gain of freshweight of cultivated hairy roots. Normally, to simplify, only 2-3 genes will be targeted. The first target genes are specific genes related to elongation, good growth of fresh biomass of hairy roots. There are *rolB* (Spena *et al*., 1987) and *GA20ox* genes. The second target gene is a housekeeping gene, which is uniformly expressed among all cells even under different experimental conditions. Normally, in majority of scientific papers it is reported that often *Actin* or *GADPH* genes as control genes for gene expression survey experiments. In this experiment, the *Actin* gene is selected for investigation. Based on the result of comparing the weights of the band expressing the target and control genes, between the sample

applying the survey conditions and the control sample, it could be conclused whether the target gene is being expressed strongly or weakly under survey conditions.

MATERIALS AND METHODS

Plant Materials

Ngoc Linh ginseng hairy roots T4 cultivars were induced by genetic transformation (Nilsson, Olsson, 1997), selected for stable growth and maintained in *in vitro* culture by the Laboratory of Department of Plant Cell Technology, Institute of Biotechnology (Pham Bich Ngoc *et al*., 2013).

B5 culture medium supplemented with 0.5 mg/L IBA; 1.0 g/L Trytophan; 7.5 g Agar. This is the optimal *in vitro* medium for Ngoc Linh ginseng root culture obtained from previous studies.

The chemicals and equipment commonly used in experiments are provided by Plant Cell Technology Department - Institute of Biotechnology.

Methods

Ngoc Linh ginseng hairy root culture

Preparation of culture medium

B5 culture medium, pH standard adjusted at 5.8 using NaOH 5M solution. The autoclave was disinfected at 117°C, 15 minutes later, supplemented with B5 vitamins, 0.5 mg/L IBA; 1.0 g/L Trytophan (sterilized using a bacterial filter) in the culture box.

PAW is used within 24 hours after activation. Use a 0.2 µm filter to sterilize the PAW before adding it to the culture media using experimental formulas such as Table 1.

Table 1. Experimental formulas (Each formula was performed with 4 flasks. The experiment was repeated 3 times)

No.	Test formula	PAW (mL/flask)	Medium (mL/flask)
1	Control	0	100
2	5%	5	95
3	10%	10	90
4	20%	20	80

Table 2. Primers using for RT-PCR of Actin, rolB and GA20ox genes in PAW treated Ngoc Linh ginseng hairy roots

Gene	F primer (5'-3')	R primer (5'-3')
Actin	ACGGGAAATCGTTCGTGACA	GACCCACCACTCAGCACAATG
rolB	CCGAGCTCTTAGGCTTCTTTCTTCAG	GCTCTAGAATGGATCCCAAATTGCTA
GA20	GCCTTCAAGTCTTTGTGGAA	GAGCTCTCACAGATCCTCTTCT

Cultivating in vitro Ngoc Linh ginseng hairy root

Five weeks old *in vitro* Ngoc Linh ginseng hairy roots were blotted dry on sterilized absorbent paper and weighed exactly 1 g in the sterile culture box and placed in a 250 mL flask containing B5 culture medium. The culture flasks are placed in a thermostatically controlled shaking cabinet at 22°C, with a shaking speed of 90 rpm. After 2 weeks of culture, the roots in the flask were transferred into a 250 mL flask containing culture media according to the experimental formulas prepared above. The culture flasks were continued to be placed in a thermostatic shaking cabinet at 22 °C, with a shaking speed of 90 rpm. After 12h, 24h, 48h, 72h, collect samples to conduct RNA extraction.

DNA extraction from Ngoc Linh ginseng hairy root samples

For Ngoc Linh ginseng root tissue, CTAB extraction method with extraction buffer containing PVP was used. The obtained DNA is used as a positive control in the PCR reaction.

Separation of total DNA: Crush 0.2 g of Ngoc Linh ginseng root tissue in a 2 mL eppendorf tube with iron balls. Add 750 µL CTAB extraction buffer (100 mM Tris- HCl pH8, 20 mM EDTA, 1.4 M NaCl, 2% CTAB, 1% PVP), incubate for 60 minutes at 65°C, mix well every 15 minutes. Add 750 µL of mixed Chloroform: Isoamylalcohol (24: 1), stir well, centrifuge at 12,000 rpm for 15 minutes, and collect the aqueous phase. Add isopropanol in a 1: 1 ratio, mix well, centrifuge at 12,000 rpm for 15 minutes, and collect DNA residue. Wash the residue 2 times with EtOH 70%. DNA drying. Dissolve the residue in 50 µL of water.

RNA extraction from Ngoc Linh ginseng hairy root tissue

Total RNA from 1 month old Ngoc Linh ginseng roots cultured on B5 medium was extracted according to TRIzol Reagents (Life Technologies). Hairy roots are ground in liquid nitrogen to a fine powder. Add 800 µL Trizol, stirring at room temperature for 10 minutes, add 200 µL Chloroform: Isoamyl alcohol (24: 1), stir gently and centrifuge 13,000 rpm for 15 minutes. Suction fluid, precipitate.

RNA with Isopropanol (1: 1 ratio), keep -20°C for 30 minutes. Centrifuge at 13,000 rpm for 10 minutes, discard the supernatant, wash the precipitate with EtOH 70%. Dry and dilute RNA in DEPC 0.01% water. The products were tested using electrophoresis on 0.8% agarose gel.

RT-PCR method

- *Reverse transcription:* Mix 1 containing following components in each 250 µL eppendorf tube: 1 µL OligoT/Hexamer.

1 - 11 µL Total RNA (5 mg/µL); add DE1-11 PC water up to a total volume of 12 µL. Incubate mix 1 at 65°C for 5 minutes, then immediately transfer to the ice. Add 8 µL mix 2 including 4 µL Reaction buffer; 2 µL dNTP mix; 1 µL Rebiloch; 1 µL Reverdard. Mix well spin down and incubate in a PCR machine by following cycle: 5 min at 25°C, 60 min at 45°C and 5 min at 70°C.

- PCR: performed with 20 - 50 ng total isolated RNA (for each reaction) and specific primers for *Actin, rolB, GA20ox* genes shown in table 2. The RT- PCR reaction volume composes of 4.5 µL deionized H_2O; 7.5 µL PCR master mix 2X; 0.5 µL each forward primer and backward primer; 2 µL cDNA template to a total volume of 15 µL. The RT-PCR was performed as following: denaturation at 94°C for 4 min, then 30 thermocycles of 20 sec denaturation for 94°C; 20 sec annealing at 55°C for *Actin* and *rolB* gene and 58°C for *GA20ox* and 40 sec elongation at 72°C. The amplification terminates with 4 min for additional elongation and stored at 16°C until product analysis by electrophoresis on 1.5% agarose gel.

RESULTS AND DISCUSSION

Quality and quantity of total DNA isolated from Ngoc Linh ginseng hairy roots

T4 Ngoc Linh ginseng hairy roots cultured *in vitro* were sampled and isolated total DNA. Total DNA extraction results were tested by electrophoresis on the 1% agarose gel, which showed that the extraction results on all samples had only a single band. The bands appear clear and bold showing that the total DNA extracted is quite intact, the rate of DNA fracture is low and can be used for PCR. The experiment was repeated 3 times and obtained the results shown in Figure 1A and Table 3A.

RNA extraction of Ngoc Linh ginseng roots

Quality and quantity of RNA extracted from Ngoc Linh ginseng hairy roots

One month T4 hairy roots cultured on B5 medium were used for RNA extraction. After extraction, the product concentration was measured (Table 3B) and 3 µL electrophoresis sample with 3 µL loading dye on 0.8% agarose gel, we obtained the results in Figure 1B showing clear three bands with sufficient quality for further analysis.

RNAs from PAW treated Ngoc Linh hairy root samples

After 12h, 24h, 48h, 72h cultivation according to the experimental formulas shown in table 1, Ngoc Linh ginseng hairy root samples were collected for RNA extraction. Quality of the obtained RNA samples were determined by electrophoresis on 0.8% agarose gel. Electrophoresis results are shown clearly three RNA bands of 28S, 16S and 5,8S, respectively (Figure 1C). The measured concentration are shown in Table 3C. PAW treatment at the concentration of 5% caused a slighly increase of RNA isolated from the same fresh biomass, only after 24 and 48 hrs. Higher PAW concentration in the culture media even cause a decrease of RNA amounts. These results proved that the quality and quantity of the RNA samples are sufficient for further analysis.

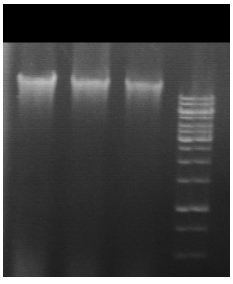


Figure 1A. Total DNA of Ngoc Linh ginseng hairy roots. Total DNA was obtained from 3 different culture flasks (1, 2,3); M = DNA1 kb marker

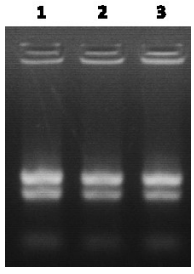


Figure1B. RNA of Ngoc Linh ginseng hairy roots. RNA was obtained from 1-3 different culture flasks (1, 2, 3). Three RNAs observed: 28S; 16S and 5.8S

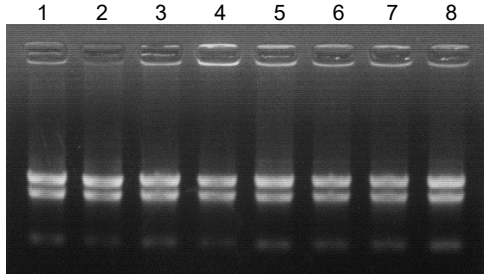


Figure1C. RNAs of Ngoc Linh ginseng hairy root samples which were treated with PAW differently in concentration (5-20%) and duration (12, 48 and 72 h). 1: Control; 2: 48 h 5%PAW; 3: 48 h 10%PAW; 4: 48 h 20%PAW; 5: Control; 6: 72 h 5%PAW; 7: 72 h 10%PAW; 8: 72 h 20%PAW. Three RNAs observed: 28S; 16S and 5.8S

Table 3A. Quality and quantity of DNA isolated from 3 different tissue samples of Ngoc Linh ginseng hairy roots

Sample	A260/280	DNA conc. (ng/ µL)
1	2,06	1237
2	2,00	620
3	1.97	460.7

Table 3B. Quality and quantity of RNA isolated from 3 different tissue samples of Ngoc Linh ginseng hairy roots

Sample	A260/280	RNA conc. (ng/ µL)
1	1.95	726.5
2	1.88	561
3	1.94	682

Table 3C. Quality and quantity of RNAs extracted from PAW treated Ngoc Linh ginseng hairy root samples

Sample	A260/280	RNA conc. (ng/ µL)	Sample	A260/280	RNA conc. (ng/ µL)
Control 12h	1.95	637.5	Control 48h	1.81	631.4
PAW 5% 12 h	1.89	606.7	PAW 5% 48h	1.96	844.1
PAW10% 12h	1.93	637.6	PAW 10%48h	1.99	967.0
PAW 20% 12h	1.97	744.3	PAW 20%48h	1,94	677.4
Control 24h	1.99	921.7	Control 72h	1.94	679.3
PAW 5% 24h	2.00	857.0	PAW 5% 72h	1.91	533.8
PAW 10% 24h	2.02	990.1	PAW 10%72h	1.91	372.4
PAW 20% 24h	1.98	849.9	PAW 20%72h	1.93	566.1

Expression of genes related to proliferation in Ngoc Linh ginseng hairy root tissues

When applying RT-PCR for the gene expression survey, total RNA from Ngoc Linh ginseng hairy root tissues need to be extracted. The RNAs are then converted to cDNA by a reverse transcription reaction. Finally, cDNA is added to the PCR reaction, which contains specific primers (table 2) of the coding gene for transcription factors.

Expression of Actin gene

The electrophoresis of the RT-PCR products is shown in Figure 2 show that there is a bold and clear band appearing in the samples of Ngoc Linh ginseng root cDNA and positive control (DNA Ngoc Linh ginseng). Particularly with the negative control sample (dH_2O), there was no occurrence of banding. The *Actin* gene gives a clear electrophoresis result, the *Actin* gene is an ideal housekeeping gene for RT-PCR experiments to evaluate the expression of related genes.

Expression of rolB gene

We see a bold and clear band appeared in samples of Ngoc Linh ginseng root cDNA and positive control (Ngoc Linh ginseng DNA) in the electrophoresis results obtained in Figure 2B, while in the negative control samples (dH_2O), no product appears. The *rolB* gene electrophoresis results in clear lines, which is an ideal expression gene for RT-PCR experiments to evaluate the expression of related genes.

Expression of GA20ox gene

Figure 2C shows that only a bold and clear line appears in the positive control sample (DNA Xoan ta gene transfer *GA20ox*), no band appears in the negative control samples (dH_2O), even no product could be formed in all cDNA samples of Ngoc Linh ginseng. The electrophoresis results of *GA20ox* gene showed no *GA20ox* expression in Ngoc Linh ginseng root. In conclusion, two target genes *Actin* and *RolB* are suitable for expression study in Ngoc Linh hairy roots using RT-PCR, while the *GA20ox* gene is not.

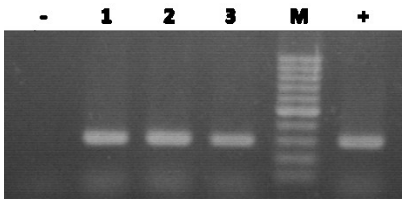


Figure 2A. RT-PCR results of Actin gene expression at transcriptional levels in Ngoc Linh ginseng root
M. marker 100 bp; (-). negative control (dH_2O); (+). positive control (DNA of Ngoc Linh ginseng); 1-3. cDNA Ngoc Linh ginseng root.

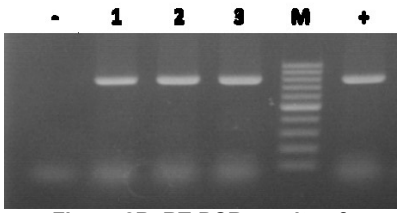


Figure 2B. RT-PCR results of expression of rolB expression at transcriptional level in Ngoc Linh ginseng root
M. marker 100 bp; (-). negative control (dH2O); (+). positive control (DNA of Ngoc Linh ginseng); 1-3. cDNA Ngoc Linh ginseng root

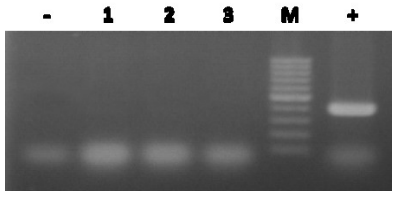


Figure 2C. RT-PCR results of GA20ox expression at transcriptional levels in Ngoc Linh ginseng root
M. marker 100 bp; (-). negative control (dH2O); (+). positive control (the DNA of our genetically modified GA20ox); 1-3. cDNA Ngoc Linh ginseng root

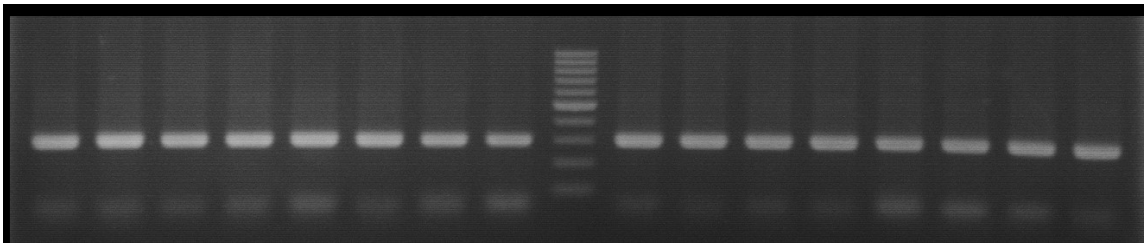
Impact of PAW treatment on the expression of genes involved in proliferation (*Actin* and *rolB*)

RNA samples have been isolated from total 16 hairy root samples treated with PAW differently in concentration (from 0 to 20%) and time (from 12 to 72 hrs.) RT-PCR using primers specific for 2 genes *Actin* and *rolB.* The electrophoresis results show that the 16 samples exhibited the *Actin* gene fairly evenly under different

experimental conditions, indicating that the housekeeping gene *Actin* is always active. Meanwhile, the expression of *rolB* gene in Figure 3B shows that the *rolB* gene was well expressed in the B5 medium supplemented with 5% PAW compared to control samples in the early stage (in the first 24 hours when PAW was added). In contrast, when supplemented with higher concentrations of PAW (10%, 20%), inhibited the expression of *rolB* expression. In general, decreasing *rolB* gene expression could be observed by increasing PAW concentrations. Over the treatment time, the *rolB* gene was most strongly expressed in samples collected after 12 h of PAW supplementation and getting weaker after 72 hrs. of PAW supplement. In conclusion, PAW treatment at 5% stimulates immediately the growth of fresh biomass combining with increasing *rolB* gene expression, while higher concentration and long lasting treatment impact negatively on the growth and gene expression. Specific band analysis software for exact determination the expression activities is going on.
New study on adding other ion (Na_2CO_3) into water during the plasma expossion creates higher concentration of radicals in PAW. Further study on the effects of these new PAWs on groth and gene expression in Ngoc Linh ginseng hairy roots cultures is going on.

Time (hrs)	12	12	12	12	24	24	24	24		48	48	48	48	72	72	72	72
%PAW	0	5	10	20	0	5	10	20	M	0	5	10	20	0	5	10	20

3A. Expression of Actine gene during PAW treatment



B. Expression of rolB gene during PAW treatment

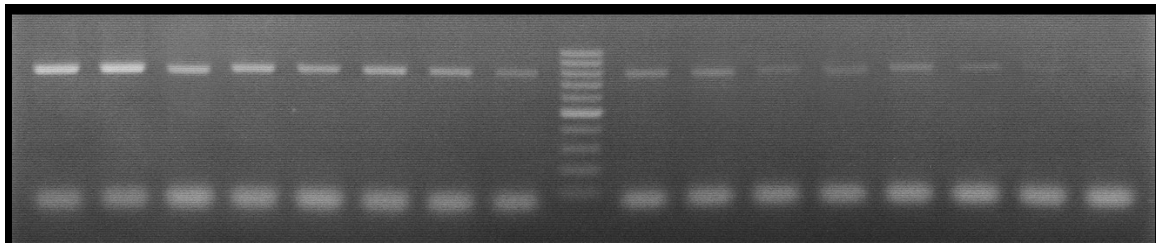


Figure 3. Results of RT-PCR examining gene expression at the transcriptional level in Ngoc Linh ginseng hairy roots treated with PAW differently in concentrations (from 0 to 20%) and time (from 12 to 72 hrs). M. marker 100 bp; A. Gen Actin; B. rolB gene

CONCLUSION

GA20ox gene proved to be not expressed in Ngoc Linh ginseng hairy roots, therefore is not suitable as target gene for expression study on PAW impact.

Housekeeping gene *Actin* is always active in culturing condition under suplementation of PAW into the culture medium.

Adding PAW at the concentration of 5% into the B5 culture medium of Ngoc Linh ginseng hairy roots stimulates the expression of *rolB* gene in the tissues, while the higher PAW concentration and the longer treatments resulted negatively impact on *rolB* gene expression. Further study on other higher radical PAW on growth and gene expression in Ngoc Linh ginseng hairy root culture is going on.

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TÁC ĐỘNG CỦA NƯỚC HOẠT HÓA PLASMA (PAW) LÊN BIỂU HIỆN MỘT SỐ GEN LIÊN QUAN ĐẾN SINH TRƯỞNG CỦA RỄ TƠ SÂM NGỌC LINH (*Panax vietnamensis* Ha et Grushv.)

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TÓM TẮT

Nghiên cứu trước đây của chúng tôi khẳng định nước hoạt hóa plasma (PAW) có tính diệt khuẩn, diệt tế bào ung thư, nhưng cũng kích thích hạt nảy mầm và gia tăng sinh khối mô tế bào nuôi cấy, trong đó chúng tôi chú ý là khả năng kích thích tăng sinh khối tươi của rễ tơ sâm Ngọc Linh nuôi cấy *invitro*. Trong nghiên cứu này chúng tôi đánh giá tác động của PAW lên biểu hiện một số gen liên quan đến sinh trưởng của rễ tơ sâm Ngọc Linh. Phương pháp được sử dụng là RT-PCR, đối tượng gen được lực chọn gồm gen *rolB*, gen *GA20ox* và gen *Actin*. Kết quả thu được cho thấy: (1) Có thể tách chiết DNA và RNA với số lượng và chất lượng tốt từ mô rễ tơ sâm Ngọc Linh nuôi cấy để sử dụng cho mục đích RT-PCR. (2) Có thể sử dụng mẫu RNA thu được và những cặp mồi đặc hiệu cho các gen *Actin*, *rolB* và *GA20ox* để tổng hợp cDNA và tiến hành RT-PCR đối với các gen trên. (3) Dưới tác động của PAW ở nồng độ tbổ sung vào mô trường nuôi cấy từ 0 đến 20% và thời gian theo dõi sau khi bổ sung PAW vảo môi trường nuôi cấy là từ 12 đến 72 giờ cho thấy biểu hiện của gen *Actin* là ổn định không thay đổi; gen *rolB* biểu hiện tăng sau 12 giờ xử lý với 5% PAW, nhưng lại giảm ở nồng độ cao hơn và thời gian sau đó, riêng gen *GA20ox* không hoạt động trong rễ tư sâm Ngọc Linh. Từ kết quả đó có thể kết luận PAW tác động rất sớm và chỉ cần nồng độ thấp (5%) để kích hoạt gen tăng sinh *rolB* trong mô rễ tư sâm Ngọc Linh. Nghiên cứu tác động lên sinh trưởng và biểu hiện gen của PAW với thay đổi các gốc hoạt hóa đang được triển khai nhằm khám phá tác động sinh học tế bào và sinh học phân tử của PAW.

Từ khóa: Nước hoạt hóa plasma, rễ tơ Sâm Ngọc Linh, biểu hiện gen *rolB*, gen *Actin* và gen *GA20ox*.

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