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Effect of GO on the formation and differentiation of tobacco leaf callus and its antioxidant capacity

Junyi Huang, Peng Feng, Nannan Chen, Ping Liu, Ping Li, Xianyan Liao*

Laboratory of Food Nutrition and Function, School of Life Science, Shanghai University, Shanghai, 200444, China.

*Corresponding author e-mail: xyliao@shu.edu.cn

Abstract. The present study was carried out to explore the effects of graphene oxide (GO) on callus induction, differentiation, metabolite biosynthesis and antioxidant capacity for tobacco leaves. Results showed that lower concentrations of GO (lower than 600 $\mu\text{g/mL}$) stimulate the differentiation of tobacco callus into buds and leaves. The content of chlorophyll, soluble sugar, soluble protein, and the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) all increased at lower concentrations of GO. In addition, the length of roots and stems of the regenerated tobacco callus also increased. However, GO induced an increase in malonaldehyde (MDA) content. The experimental results could provide valuable reference for other researches about interactions between carbon-based nanomaterials and plant in callus formation differentiation, metabolite biosynthesis and nanometer pollution.

1. Induction

Increasing applications of carbon-based nanomaterials in the field of plant production and food science have raised concerns about their effects on living organisms [1]. Some researchers aimed at the effect of carbon-based nanomaterials on plants and they have obtained some results including positive and negative [2-4]. Cell and tissue culture systems have been considered for the selection of plant stress factors such as salinity and drought [5].

Javed et al. [6] found that both 100 mg/L ZnO NPs and 10 mg/L CuO NPs could increase the total phenolic content, total flavonoid content and total antioxidant capacity of stevia callus. Few researches focused on the effects of carbon-based nanomaterials on callus. In this study, the potential effects of GO at different concentrations on callus induction and metabolites biosynthesis, activities of antioxidant enzymes were investigated. The results showed that lower concentrations of GO (less than 600 $\mu\text{g/mL}$) are beneficial for callus formation and differentiation.

2. Materials and methods

2.1. Synthesis and characterization of GO

GO was synthesized according to the method of Wang et al. [7]. All the characteristics of GO were reported by Chen et al., our previous research [8]. GO had a specific peak at 230 nm. And its maximum fluorescence emission wavelength was 472 nm. The thickness was about 1nm. The results of X-ray diffraction (XRD) showed that the diffraction angle of GO was 10.8 degree.



2.2. Effect of GO on the formation and differentiation of tobacco leaf callus

The leaves of tobacco as an explant were rinsed with tap water, disinfected with 70% ethanol for 30 s and 5% sodium hypochlorite for 5 min, and then rinsed 3 times with sterile water. After the main veins and leaf edges were removed by a scalpel, the leaves were cut into 1 cm² squares and inoculated onto the induction medium. The MS medium is consisted of 1 mg/mL 6-benzylaminopurine (6-BA), 0.5 mg/L 1-naphthylacetic acid (NAA), 3% sucrose and 0.75% agar in final concentration, the pH of the medium was adjusted to 5.8, and the medium was autoclaved at 121°C for 20 min. GO was added before the sterilized medium was cooled (about 50°C), and finally a solid medium containing different concentrations of GO was obtained (25, 50, 100, 200, 300, 400, 500, 700, 800, 900, 1000 and 1100 µg/mL). Three slices of fresh tobacco leaves were introduced into each bottle and then transferred to a light incubator for culture. The light incubator maintained a temperature of 25°C ± 2°C, 16/8 light/dark photoperiod cycle, and 2000 µmol m⁻²s⁻¹ of light intensity.

The growth of tobacco callus was observed and photographed every 7 days. The number of splitting of callus, the fresh weight (W_F) and the dry weight (W_D, 65°C bake for 24 h) under different concentrations of GO was calculated after 28 days cultivation. The dry matter content was calculated using the following formula (1):

$$\text{Dry matter content} = W_D / W_F \quad (1)$$

2.3. Chlorophyll estimation

Chlorophyll content was determined by the method of Arnon et al. [9]. 0.2 g tissue was collected, rinsed with distilled water and sucked dry, then cut into small pieces and placed in test tubes. 10 mL of acetone-ethanol extract (V/V = 1:1) was added to the tube, and diluted to 25 mL after shaking for 14 h in the dark. The absorbance of supernatant was measured at 645 and 663 nm wavelength. Results were calculated by fresh weight (FW). The chlorophyll content was calculated according to the following formula:

$$\text{Chlorophyll a (Chl a) concentration (g/L)} = 0.0127A_{663} - 0.00269A_{645} \quad (2)$$

$$\text{Chlorophyll b (Chl b) concentration (g/L)} = 0.0229A_{645} - 0.00468A_{663} \quad (3)$$

$$\text{Total chlorophyll concentration} = \text{Chl a} + \text{Chl b} \quad (4)$$

2.4. Soluble sugar estimation

Soluble sugar content was determined by the method of Yang et al. [10]. 0.1 g dry callus sample were transferred to the centrifuge tube, and 4 mL 80% ethanol was add, 80°C water bath for 30 min followed by 6,000×g centrifugation for 10 min. And then repeated the above procedure so that the sample could be extracted twice. The supernatant was filtered and then made up to 10 mL. 0.2 mL extract were mixed with 5 mL anthrone test solution, and then they were incubated in the boiling water bath for 10 min. After cooling down to the room temperature, the absorbance at 625 nm was measured. The soluble sugar content was calculated using the following formula (5):

$$\text{Soluble sugar content (mg/g DW)} = (C \times V_T) / (1000 \times W \times V_i) \quad (5)$$

In the formula, C is the mass of the soluble sugar (g) obtained from the standard linear equation; V_T is the volume of the extract (mL); V_i is the volume of the assay fluid (mL); DW is the dry weight of the sample (g).

2.5. Determination of soluble protein and antioxidant enzyme activities

The kits to analyse soluble protein, SOD, POD and CAT were purchased from Jiancheng Bioengineering Co. Ltd. (Nanjing, China). Callus were collected every 7 days during the culture period and then were

homogenated by ultrasonic, after that the callus homogenate (20%) was centrifuged at $6,000\times g$ for 10 min. The supernatant was used to measure the content of protein, and the activities of SOD, POD and CAT according to the manufacturer's instructions. Three independent experiments were performed for each condition.

2.6. Lipid peroxidation level estimation

The MDA content was measured as described by Stewart et al. [11]. 0.2 g callus sample were homogenized in 2 mL of 10% trichloroacetic acid (TCA) and centrifuged at $10,000\times g$ for 10 min. 1.8 mL supernatant (control group added an equal volume of 10% TCA) was mixed with 1.8 mL of 0.6% TCA, heated at 100°C for 15 min, then cooled in an ice bath and centrifuged again ($10,000\times g$ for 10 min). Absorbance of supernatant was measured at 450 nm, 532 nm and 600 nm. MDA content was calculated using the following formula (6):

$$\text{MDA content } (\mu\text{mol/g}) = [6.45 \times (D_{532} - D_{600}) - 0.56D_{450}] \times V \div M \quad (6)$$

Where V is the extract volume (mL), and M is the weight of fresh leaves (mg).

2.7. Effects of GO on roots and stems of tobacco callus regeneration system

After 21 days culture in MS medium containing different concentrations of GO, the buds in each sample were taken out and inoculated into the corresponding concentration of rooting medium. The rooting medium was consisted of $1/2$ MS, 1.5% sucrose and 0.75% agar. And then they were transferred to a light incubator for 20 days cultivation. The growth of the roots and stems was observed and the length of the stems was measured.

3. Results and discussion

3.1. Effects of GO on the growth and differentiation of tobacco callus

Fig. 1 shows the growth and differentiation of callus, and Fig. 2 shows the number of differentiated buds in each sample after 28 days culture. Callus exposed to $25 \mu\text{g/mL}$ GO was more bulky and loose, and the division of callus was inhibited by higher concentrations of GO ($50 \mu\text{g/mL}$ GO and above). After 21 days culture, the appropriate concentrations of GO ($25\text{--}600 \mu\text{g/mL}$) promoted callus differentiation into shoots and leaves compared with the control. When the concentration of GO was $0\text{--}300 \mu\text{g/mL}$, the promotion of GO on callus differentiation was positively correlated with GO concentration, and lateral roots were observed. The $300 \mu\text{g/mL}$ GO exposed group was found to have the highest number of buds, which was 2.67 times that of the control. When the GO concentration was in the range of $300\text{--}600 \mu\text{g/mL}$, the promotion of GO on callus differentiation was found to be negatively correlated with GO concentration, and only buds were found but no roots. The results of GO stimulated callus differentiation may be attributed to the effect of GO on hormone levels in callus [12].

Bud and leaf differentiation of tobacco callus in high concentration group ($600\text{--}900 \mu\text{g/mL}$ GO) were inhibited, and the inhibitory effect showed a dose-dependent effect. Tobacco callus was found to differentiate into buds without leaves. When concentrations of GO was higher than $900 \mu\text{g/mL}$, tobacco leaves were found to gradually die, which may be due to the toxicity of high concentration of GO.

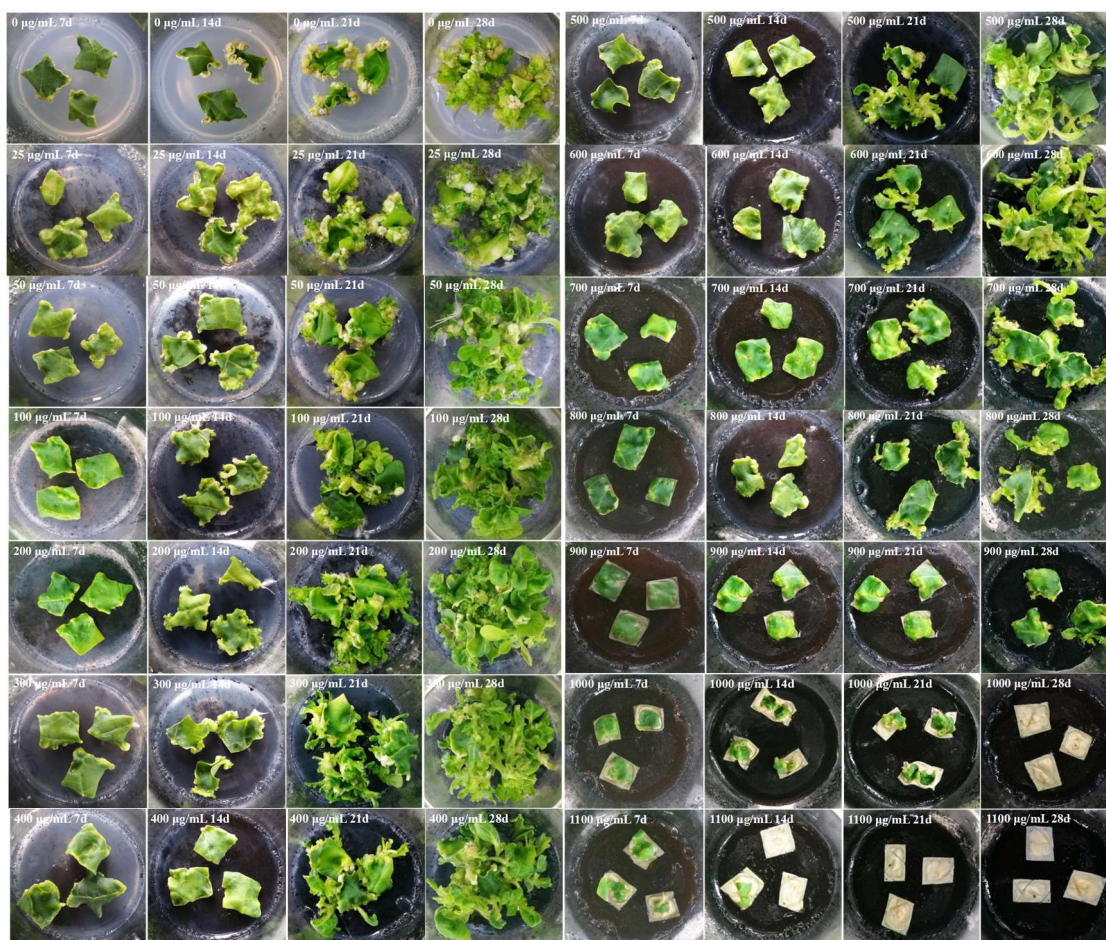


Fig 1. Effect of GO on tobacco callus formation and differentiation.

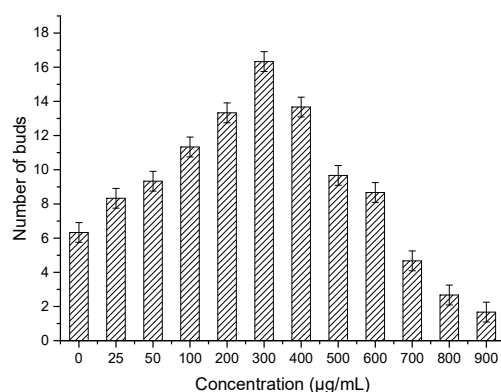


Fig 2. The effect of GO on the number of buds differentiated from tobacco callus (after 28 days culture).

3.2. Dry matter and chlorophyll content

Fig. 3A shows the dry weight content of samples exposed to different concentrations of GO after 28 days culture. When the concentration of GO was from 25 µg/mL to 500 µg/mL, the dry matter content was found to increase as the GO concentration increased. The dry matter content of the 300-500 µg/mL GQ exposure group was found to be higher than that of the control, which may be due to the formation

of buds and leaves. The dry matter content of tobacco callus decreased gradually after the GO concentration was higher than 500 $\mu\text{g/mL}$.

As shown in Fig. 3B, when the concentration of GO was 25-400 $\mu\text{g/mL}$, the content of chlorophyll was found to increase as the concentration of GO increased. The highest chlorophyll content was found in the 400 $\mu\text{g/mL}$ GO exposure group (104.28 mg/g FW), which was 15.45 times of the control group. The chlorophyll content was found to gradually decrease as the GO concentration continued to increase (above 400 $\mu\text{g/mL}$).

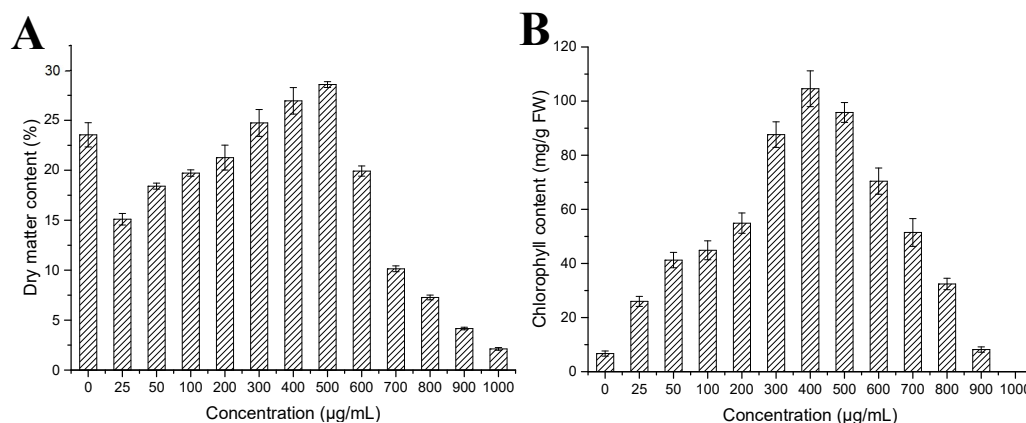


Fig. 3 The effect of GO on dry matter and chlorophyll content of tobacco callus (after 28 days culture). A: Dry matter; B: Chlorophyll

3.3. Soluble sugar and soluble protein content

Soluble sugar is not only a source of energy for plants, but also an important osmotic regulator, which has a stabilizing effect on cell membranes and protoplasts [13]. When the concentration of GO was lower than 800 $\mu\text{g/mL}$, the soluble sugar content gradually increased with the extension of the culture time (Fig. 4A). After 28 days culture, the highest soluble sugar content (10.28 mg/g DW) was found in the 500 $\mu\text{g/mL}$ GO exposure group, which was 1.51 times of the control. The content of soluble sugar in the tissue decreased gradually when the concentrations of GO was higher than 900 $\mu\text{g/mL}$, which indicated that high concentrations of GO had toxic effects on tobacco callus.

Most of the soluble proteins in plants are involved in various metabolic enzymes, and their content can reflect the metabolic status of plants [14]. As shown in Fig. 4B, when the exposure concentration was less than 600 $\mu\text{g/mL}$, the content of soluble protein in tobacco callus increased as the GO concentration increased. Increased soluble protein content was beneficial to improve the tolerance of callus. When the GO concentration was less than 600 $\mu\text{g/mL}$, the content of soluble protein was positively correlated with the GO concentration. After 28 days culture, the maximum soluble protein content (1.6 mg/g DW) was found in the 600 $\mu\text{g/mL}$ GO exposure group, which was 5.16 times of the control. The soluble protein content in the high concentration exposed group (700-900 $\mu\text{g/mL}$ GO) was still higher than that of the control, but the soluble protein content was negatively correlated with the GO concentration. This may be due to the inhibition of protein synthesis by high concentrations of GO.

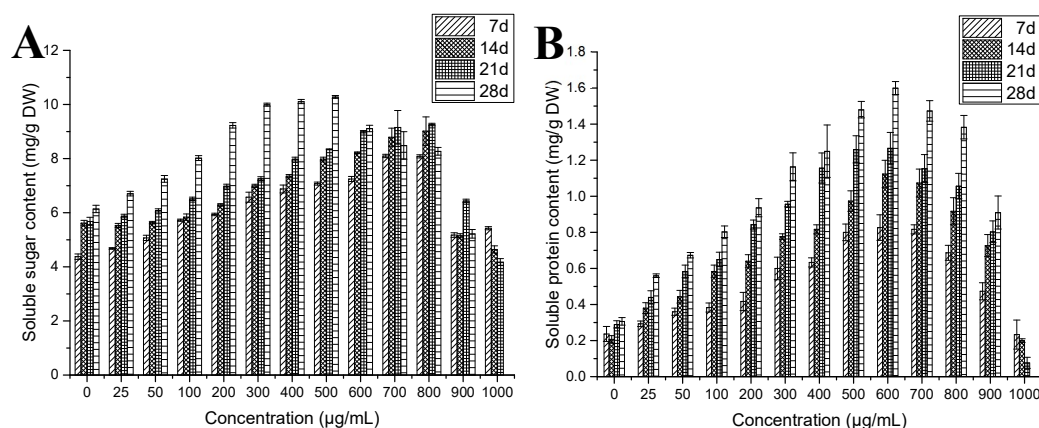


Fig. 4 The effects of GO on content of soluble sugar and soluble protein in tobacco callus. A: Soluble sugar; B: Soluble sugar

3.4. Antioxidant activity

SOD is one of the most important antioxidant enzymes in plants, which can remove excess reactive oxygen species in time and improve the antioxidant capacity of plants. As shown in Fig. 5A, the activities of SOD in the callus of the low concentration exposure group (25-500 µg/mL) was slightly increased compared with the control. After 21 days culture, the maximum SOD activity was found in the 500 µg/mL GO exposure group (404.07 U/mg prot), which was 1.18 times of the control. When the exposed concentration of GO was up to at 500 µg/mL, the SOD activity of the callus was negatively correlated with the GO concentration. After 21 days culture, the activity of SOD in the callus of 1000 µg/mL GO treatment group was only 133.64 U/mg prot, which was 38.86% of the control.

POD is an antioxidant enzyme that is widely present in plants and its activity reflects the ability of plants to remove peroxides when they feel stress. As shown in Fig. 5B, as the GO concentration increased, the POD activity first increased and then decreased. After 28 days culture, the maximum active POD (217.42 U/mg prot) was found in the 500 µg/mL GO exposure group, which was 1.32 times of the control. The activities of POD decreased gradually when the concentration of GO was higher than 500 µg/mL.

CAT can promote the decomposition of H₂O₂ into molecular oxygen and water, thereby protecting tissues from H₂O₂ poisoning. As shown in Fig. 5C, when the concentration of GO was less than 900 µg/mL, the activity of CAT was positively correlated with the concentration of GO. After 28 days culture, the maximum CAT activity was found in the 700 µg/mL GO exposure group, which was 4.43 times of the control. High concentrations of GO (1000 µg/mL) were found to inhibit CAT activity. CAT was found to be more sensitive to GO than SOD and POD.

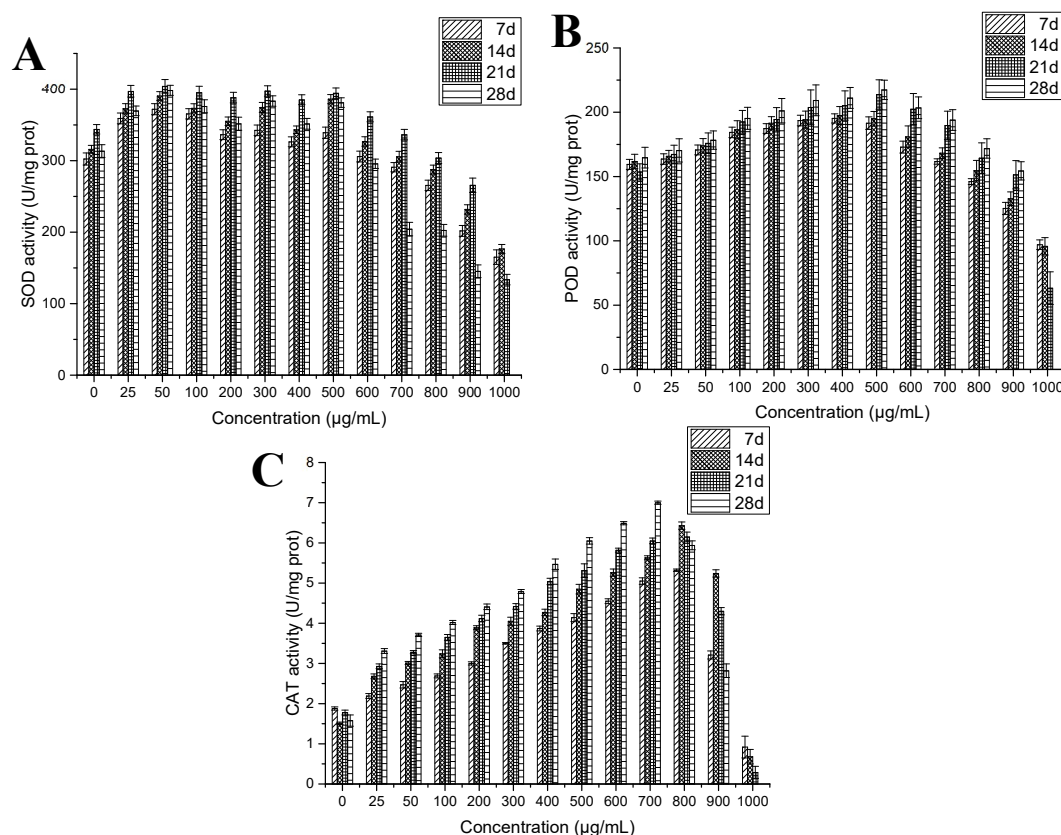


Fig. 5 Effect of GO on antioxidant enzyme activity in tobacco callus. A: SOD; B: POD; C: CAT

3.5. Lipid peroxidation level

In the study of plant senescence physiology and resistance physiology, MDA content is a common indicator for evaluating the degree of membrane lipid peroxidation. As shown in Fig. 6, as the culture time extend and the GO concentration increased, the MDA content in the tissue gradually increased. After 21 days culture, the highest level of MDA (3.01 mmol/g FW) was found in the 1000 µg/mL GO exposure group, which was 5.38 times of the control. The results showed that high concentrations of GO led to an increase in MDA content.

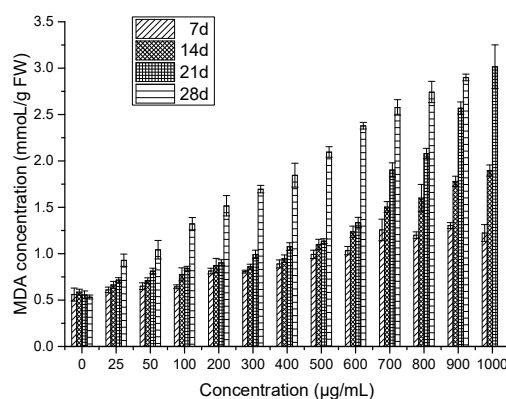


Fig. 6 The effects of GO on content of MDA in tobacco callus.

3.6. Effects of GO on the roots and stems of tobacco callus regeneration system

Fig. 7 showed the effect of different concentrations of GO on roots of tobacco callus regeneration system. It can be seen from the fig. 7 that the control group has a large number of roots and the roots are healthy. Low concentrations of GO (25-300 $\mu\text{g/mL}$) promoted root elongation, but the number of roots in these exposed groups was less than that of the control. The number of roots was significantly reduced when the concentrations of GO was higher than 300 $\mu\text{g/mL}$. Root formation of the tobacco callus regeneration system was completely inhibited by GO at a concentration of 700 $\mu\text{g/mL}$ or higher.

Fig. 8 and Fig. 9 showed the effect of different concentrations of GO on stems of tobacco callus regeneration system. Compared to the control, concentrations of GO (less than 600 $\mu\text{g/mL}$) promoted the elongation of the stem. When the concentrations of GO was less than 400 $\mu\text{g/mL}$, the elongation of the stem was positively correlated with the concentrations of GO. At higher concentrations of GO (above 400 $\mu\text{g/mL}$), the elongation of the stem was negatively correlated with the concentrations of GO. The length of stem reached the maximum at the 400 $\mu\text{g/mL}$ GO (8.35 cm), which was 2.96 times of control. High concentrations of GO (above 600 $\mu\text{g/mL}$) inhibited stem length. The tobacco plants in the 900 $\mu\text{g/mL}$ GO exposed group had the smallest stem (1.13 cm), which was 40.07% of the control.



Fig. 7 Effect of GO on the root of tobacco callus regeneration system (after 21 days

Fig. 8 Effect of GO on the stem of tobacco callus regeneration system (after 21 days

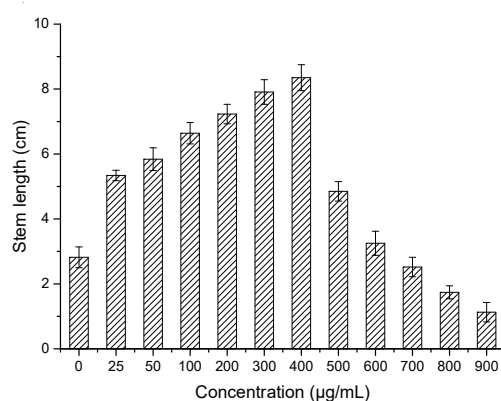


Fig. 9 Effect of GO on the length of stem of tobacco callus regeneration system (after 21 days culture).

4. Conclusion

This work provides evidence that the optimized use of GO in MS medium can be used as an effective inducer to enhance the differentiation of tobacco callus into shoots and leaves. The content of chlorophyll, soluble sugar, soluble protein, and antioxidase activity all increased in the treatment of lower concentrations of GO. In addition, the growth of the root and stem length of the tobacco callus

regeneration system was also enhanced. Higher concentrations of GO exhibited a toxicity effect to tobacco callus. Therefore, we recommend using low concentrations of GO (less than 300 µg/mL) as a new tool to improve tobacco callus differentiation.

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