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Original article

Effects of exogenous 6-BA and NAA on growth and contents of medicinal ingredient of *Phellodendron chinense* seedlings

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ABSTRACT

Using *Phellodendron chinense* seedlings as material, and treated with different concentrations of exogenous 6-Benzylaminopurine (6-BA) and α-naphthyacetic acid (NAA), then observed the growth status. Furthermore, we detected the contents of chlorophyll and soluble sugar, the activities of antioxidases by spectrophotometry, and determined the contents of secondary metabolite by high performance liquid chromatograph. The results showed that different concentrations of exogenous 6-BA increases the fresh weights and plant heights of *Phellodendron chinense* seedlings, and enhances the contents of chlorophyll and soluble sugar. NAA promoted growth, but deduced the contents of soluble sugar. Compared with control, culturing for 40 d, proper concentrations 6-BA enhanced the activity levels of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), proper concentrations NAA increased the activity levels of SOD and CAT, but decreased the levels of POD compared with CK. Suitable concentrations 6-BA enhanced contents of berberine, phellodendrine and palmatine in stems, proper concentrations NAA increased contents of berberine and phellodendrine, but deduced contents of palmatine compared with CK. Based on these results, we concluded that the exogenous 6-BA and NAA had key regulation on the growth and contents of medicinal ingredient of *Phellodendron chinense* seedlings.

1. Introduction

Phellodendron amurense is a traditional medicinal plant, belong to *Phellodendron Rupr* species, Rutaceae genus. The *Phellodendron amurense* has two species, named as *Phellodendron chinense* Schneid and *Phellodendron amurense* Rupr, and distributes in northeastern area, Sichuan and hunan province (Xian et al., 2014; Upadhyay et al., 2017; Razali and Said, 2017; Gao et al., 2017). There are many medicinal ingredients in root, stem and leaf of *Phellodendron amurense*, such as alkaloid, flavonoid and sterols. These compounds have efficiency to heat-clearing, detoxify, analgesia, diminish inflammation and reducing blood sugar, and widely used to cure dysentery, tetter and arthrolithiasis diseases (Yang et al., 2005; Garcia et al., 2006; Li et al., 2015; Swanson et al., 2015; da Silva et al., 2016; Li et al., 2017). Due to increase in demand, the wild resource of *Phellodendron amurense* was severely cut down, and resulted in shortage of supply in market. So, carrying out artificial cultivation and improving contents of medicinal ingredients is a convenient way to satisfy requirement. The growth and secondary metabolite of *Phellodendron amurense* is regulated by plant growth regulator and other factors. The previous results showed that the plant growth regulator controlled the growth and synthesis and accumulation of secondary metabolite in plant, especially traditional chinese medicinal herb and xyloptyta (He and Shi, 2014; Salerno et al., 2017; Halim and Phang, 2017). However, the effects of 6-Benzylaminopurine (6-BA) and α-naphylacetic acid (NAA) on growth and contents of medicinal ingredients of *Phellodendron chinense* are unknown. Here, we used 6-BA and NAA solution to treat seedling and determine the biomass, contents of chlorophyll and soluble sugar, activity levels of antioxidase, detect the contents of berberine, phellodendrine and palmatine in stems (Arshadullah et al., 2017; Kumruzzaman and Sarker, 2017). Our results indicated that exogenous 6-BA and NAA promote the growth, enhance enzyme levels of SOD and CAT, regulated the synthesis and accumulation of medicinal ingredient of *Phellodendron chinense*.

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2. Materials and methods

2.1. Material and culture

Using the uniformity growth seedlings of *Phellodendron chinense* as material, and sprayed its leaves with 6-BA or NAA solution (dissolved in Hoagland solution), the concentrations were 0, 10, 20 and 30 mg/L. The spraying time was 17:00–19:00 for 10 day. The control group was sprayed with Hoagland solution. Each group included three seedlings.

2.2. Determination biomass

When *Phellodendron chinense* seedling culturing on 0, 10, 20, 30 and 40th day, the plant height, weight of roots, stems and leaves were measured and weighted. The collected material was used to determined contents of chulirophyll and soluble sugar and detected the contents of medicinal composition.

2.3. Measurement contents of chlorophyll and soluble sugar

The detection of chlorophyll content and soluble sugar of *Phellodendron chinense* referred to previous methods (Liu et al., 2017; Meng et al., 2017).

2.4. Determination activities of antioxidase

The determination of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) activities of *Phellodendron chinense* referred to previous methods (Du et al., 2017).

2.5. Detection contents of medicinal compositions

The extract and detection of medicinal compositions of *Phellodendron chinense* referred to the methods (Xian et al., 2014).

3. Results

3.1. Regulation growth of Phellodendron chinense seedlings

During culturing period of 0–40 d, the fresh weight of *Phellodendron chinense* roots from control (CK) was gradually increased, and reached to maximal value at 40 d, about 4.2 g. Culturing for 40 d, the fresh weights of roots treated with 10 mg/L, 20 mg/L and 30 mg/L 6-BA were reached to peak values, about 6.8 g, 5.7 g and 5.1 g, and were 1.62-, 1.36- and 1.21-fold compared with CK (Fig. 1). When culturing on 40th d, the fresh weights of stems from CK was maximized, about 1.6 g. Meanwhile, the fresh weights of stems from *Phellodendron chinense* seedlings which treated with 10 mg/L, 20 mg/L and 30 mg/L 6-BA were reached to peach values, about 3.0 g, 2.6 g and 2.5 g, and were enhanced by 86%, 63% and 56% compared with CK (Fig. 1B). During culturing period of 0–40 d, the fresh weights of leaves from CK and 6-BA treated seedlings were gradually increased, the fresh weight of leaves spraying by different concentrations 6-BA were increased by 79.22%, 59.74% and 48.05% compared with CK at 40 d (Fig. 1C). During whole culturing period, we observed that the plant heights of *Phellodendron chinense* seedlings were gradually raised, the plant heights of *Phellodendron chinense* seedlings under 10 mg/L, 20 mg/L and 30 mg/L 6-BA treatment were reached to peak values at 40 d, and enhanced by 0.52-, 0.51-, and 0.48-fold compared with CK (Fig. 1D). Based on these results, we concluded that exogenous 6-BA promote growth of *Phellodendron chinense* seedlings.

To study the effect of NAA on growth of *Phellodendron chinense* seedlings, we used the NAA solution to spraying the leaves. During the culturing period of 0–40 d, the fresh weights of roots, stems and leaves of *Phellodendron chinense* seedlings were continued increased, and reached to peak values on 40th d. Culturing for 40 d, the fresh weights of roots which treated with 0, 10, 20 and 30 mg/L NAA solution were 4.21 g, 5.50 g, 5.96 g and 4.98 g, and enhanced by 30.49%, 41.55% and 18.27% compared with CK. Compared with CK, the fresh weights of stems under 10, 20 and 30 mg/L NAA treatment were 2.11 g, 1.76 g and 1.40 g, and increased by 0.35-, 0.13- and −0.10-fold. Culturing for 40 d, the fresh weights of leaves treated with NAA were enhanced by 21.06%, 25.86% and −10.22%. Furthermore, all different concentrations NAA raised the plant heights of *Phellodendron chinense* seedlings. Culturing for 40 d, the plant heights of seedlings under NAA treatment were increased by 38.42%, 15.99% and 13.79% compared with CK. These results indicated that low concentration NAA promoted growth of *Phellodendron chinense* seedlings.

3.2. Detection contents of chlorophyll and soluble sugar

Compared with initial stage, the contents of chlorophyll in leaves from CK and 6-BA treated seedlings were increased. Culturing for 40 d, the content of chlorophyll in leaves from CK was 1.63 mg/g FW, enhanced by 84.89% compared with initial stage. Culturing for 40 d, the contents of chlorophyll in leaves of *Phellodendron chinense* seedlings under 10, 20 and 30 mg/L 6-BA treatment were increased by 20.25%, −6.13% and −1.23% compared with CK (Fig. 2A). Meanwhile, the contents of soluble sugar in leaves treated by 10 mg/L, 20 mg/L and 30 mg/L 6-BA were enhanced by −10.82%, −26.14% and 13.78% compared with CK at 40 d (Fig. 2B).

During whole culturing period, the contents of chlorophyll in leaves of *Phellodendron chinense* seedlings treated by NAA or not were increased compared with initial stage. Culturing for 40 d, the content of chlorophyll in leaves treated by 10 mg/L and 30 mg/L NAA were enhanced by 18.16% and 20.61%, but its content in leaves with 20 mg/L NAA treatment was reduced by 4.87% (Fig. 2C). Culturing for 40 d, the contents of soluble sugar in leaves of *Phellodendron chinense* seedlings were increased compared with initial stage. The contents of soluble sugar in leaves treated by 10 mg/L and 20 mg/L NAA were no significant difference compared with CK at 40 d, but the content of soluble sugar treated by 30 mg/L NAA was reduced by 25.90% compared with CK (Fig. 2D). Based on these results, we concluded that exogenous 6-BA and NAA has key regulation to the contents of chlorophyll and soluble sugar of *Phellodendron chinense* seedlings.

3.3. Regulation activity levels of antioxidase

To investigate the effects of 6-BA and NAA on the activities of antioxidase, we determined the enzyme levels by spectrophotometer. The results showed that the activity level of SOD in leaves from CK was reached to peak at 20 d, and then dropped to the minimum at 40 d. The maximum of SOD in leaves treating with 10 mg/L, 20 mg/L and 30 mg/L 6-BA were appeared at 20 d, 30 d and 30 d, and the activity levels were enhanced by 1.48-, 1.66- and 0.38-fold compared with CK at 40 d (Fig. 3A). The levels of POD activities from CK was reached to peak at 20 d, and then reduced with prolongation of culture time. Culturing for 40 d, the Levels of POD activities in leaves treating by 10 mg/L, 20 mg/L and 30 mg/L 6-BA were enhanced by 0.59-, 1.01- and −0.78-fold compared with CK (Fig. 3B). The peak times of CAT activities were appeared at 20 d, 30 d, 40 d and 10 d. Culturing for 40 d, the levels of CAT treatment by 10 mg/L, 20 mg/L and 30 mg/L 6-BA were enhanced by 0.50-, 1.74- and −0.02-fold compared with CK, and were increased by 1.32-, 3.26- and 0.53-fold compared with activity levels of initial stage (Fig. 3C).

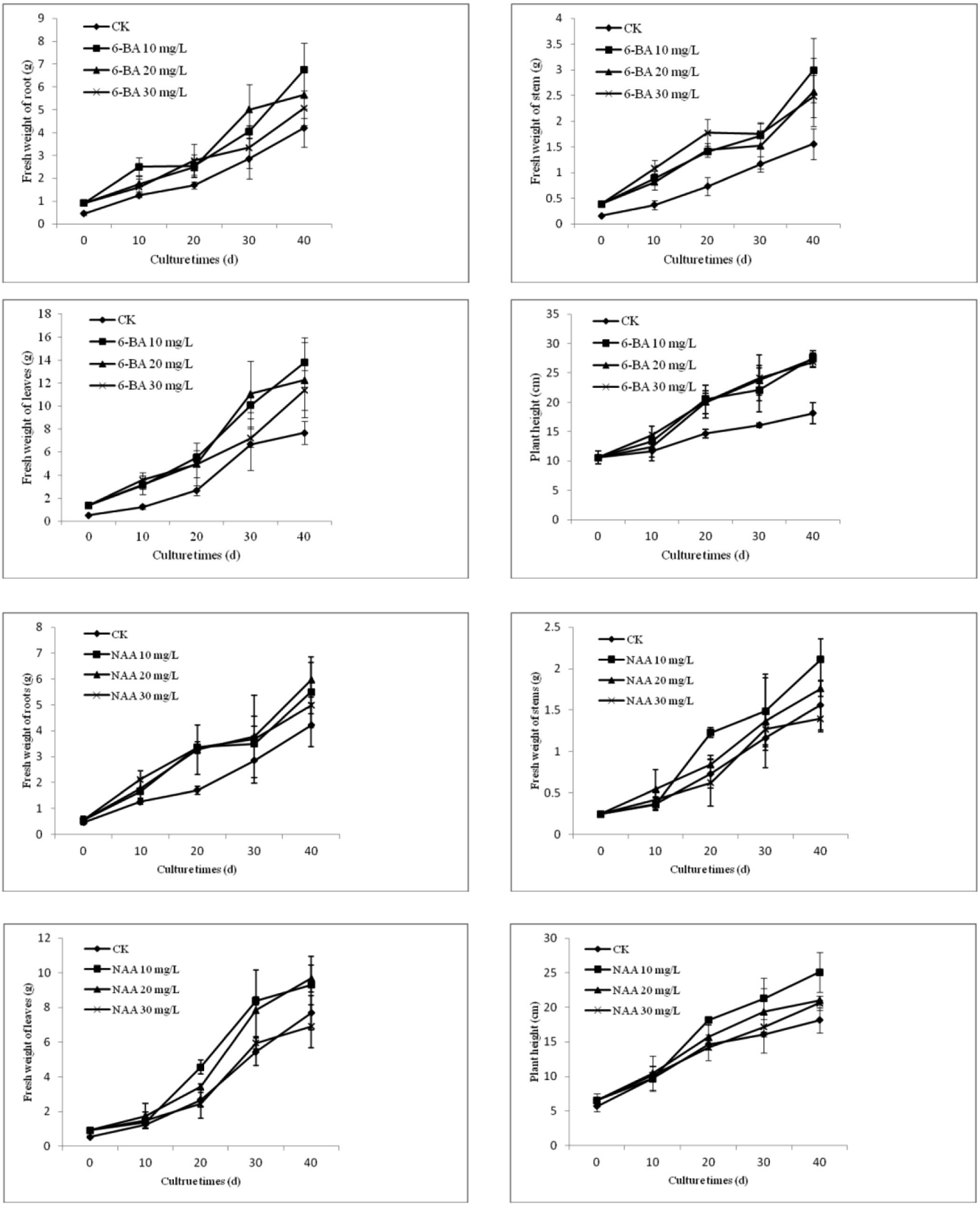
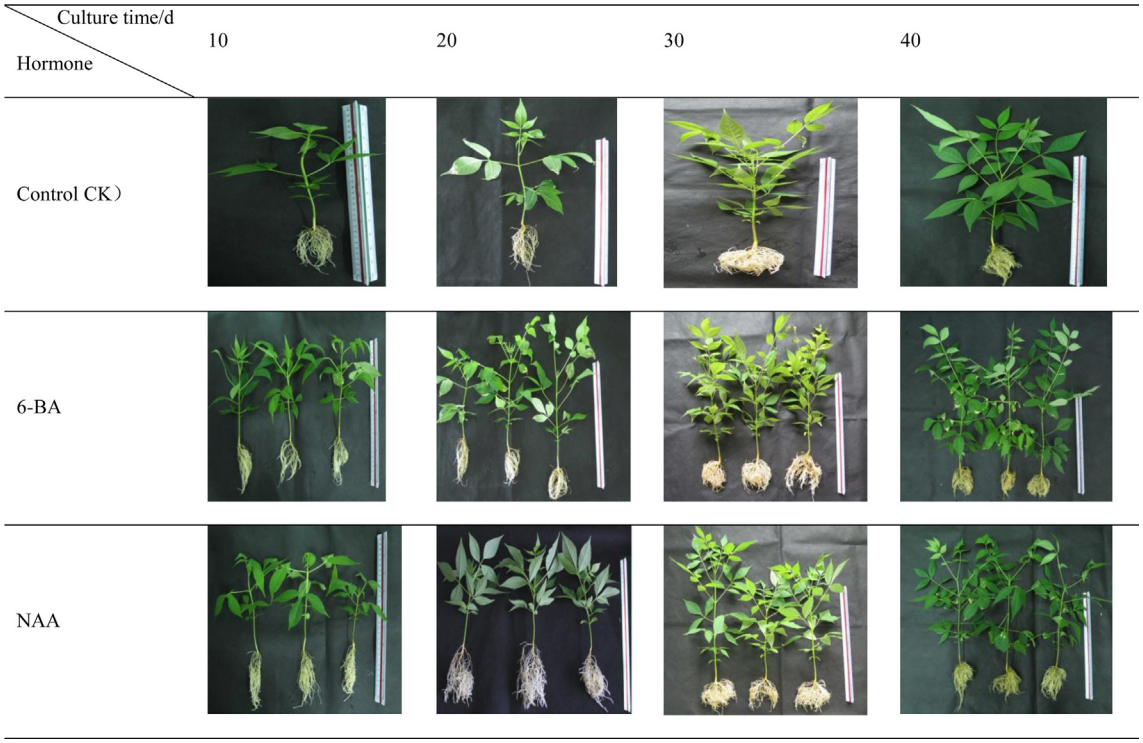


Fig. 1. Effects of exogenous 6-BA and NAA on the growth of *Phellodendron chinense* seedlings.

The NAA had important regulation to activities of antioxidase. The peak times of SOD activities in leaves of *Phellodendron chinense* seedlings treatment with different concentrations NAA were appeared at 20 d, 20 d, 20 d and 30 d. Culturing for 40 d, the enzyme levels of SOD treating by 10 mg/L, 20 mg/L and 30 mg/L NAA were enhanced by 1.07-, 0.74- and 1.46-fold compared with CK (Fig. 3D). The maximum of POD activities of *Phellodendron chinense* seedlings were appeared at 20 d, 30 d, 20 d and 20 d.



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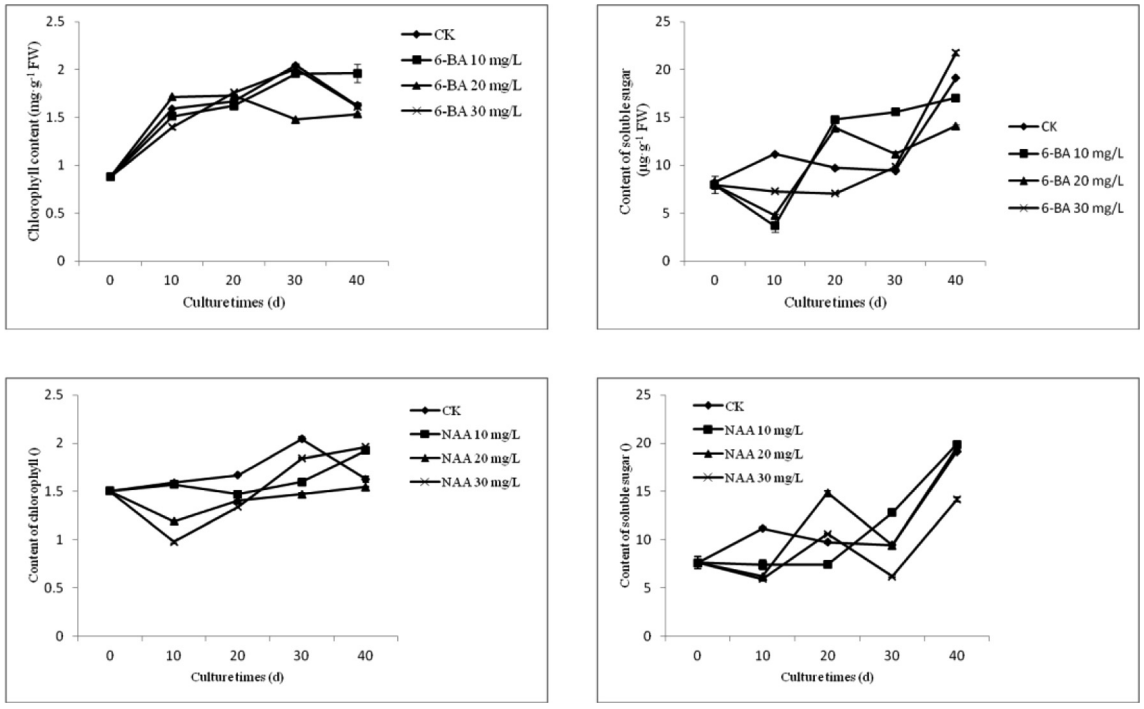

Fig. 2. Effects of exogenous 6-BA and NAA on the contents of chlorophyll and soluble sugar in *Phellodendron chinense* seedlings.

However, the enzyme levels of POD under 10 mg/L, 20 mg/L and 30 mg/L NAA treatment were 19.18, 88.04 and 57.90 percent of CK at 40 d (Fig. 3E). The CAT levels were reached to the peak values at 20 d, 40 d, 40 d and 10 d. Culturing for 40 d, the enzyme levels of CAT treated by 10 mg/L and 20 mg/L NAA were enhanced 0.84- and 1.95-fold compared with CK, but the level of CAT activity treated by 30 mg/L NAA reduced by 26.95% (Fig. 3 F). These results indicated that low concentration 6-BA and NAA enhance the activity levels of antioxidase.

3.4. Determination contents of medicinal ingredients

To investigate the effects of exogenous 6-BA and NAA on the synthesis and accumulation of medicinal ingredients in stems of

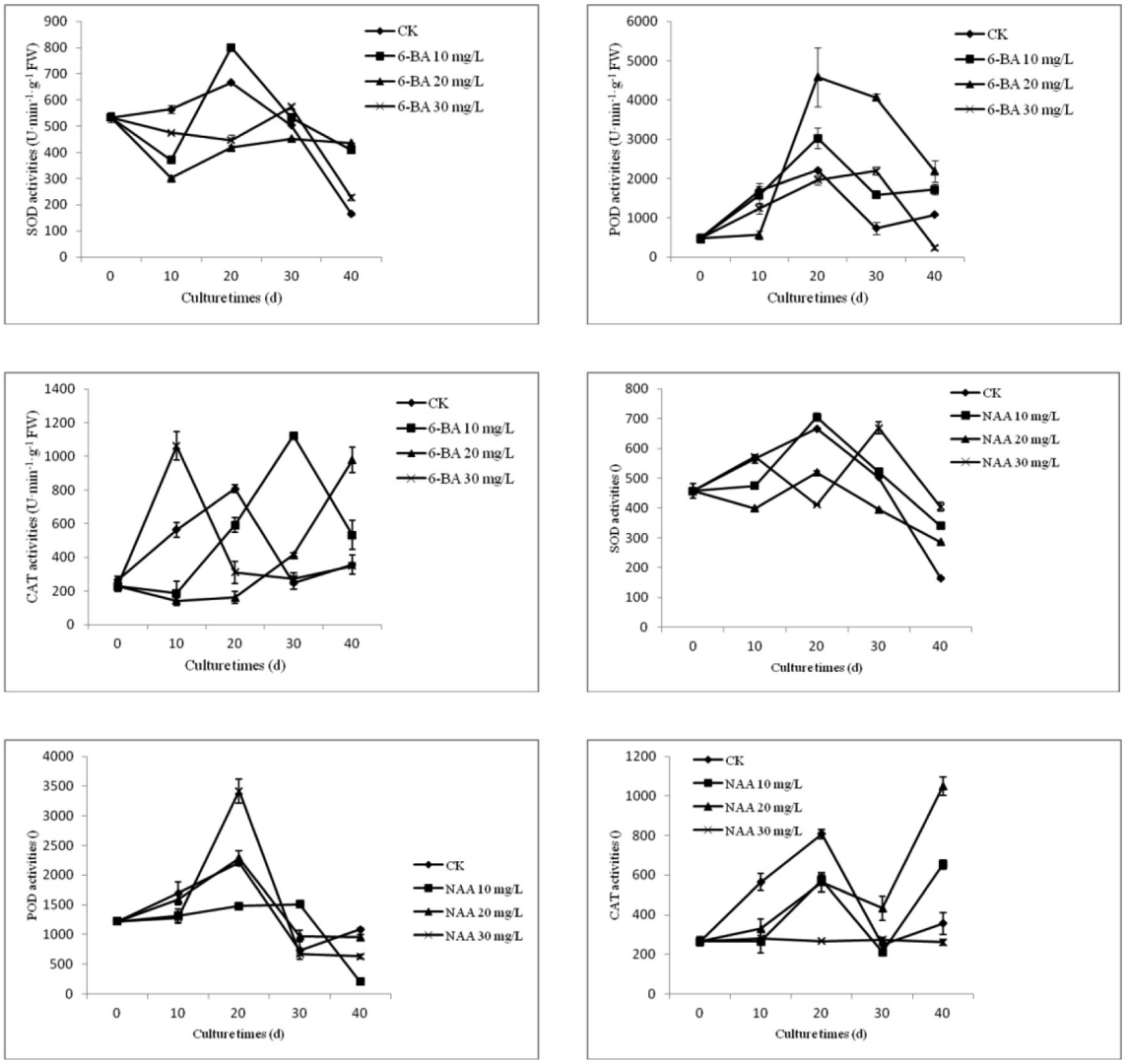

Fig. 3. Effects of exogenous 6-BA and NAA on activities of antioxidase of *Phellodendron chinense* seedlings.

Phellodendron chinense seedlings, we extracted and detected the contents of berberine, phellodendrine and palmatine by high performance liquid chromatography (HPLC). During the culturing period of 0–40 d, the contents of berberine in stems of *Phellodendron chinense* seedlings which treated by different concentrations 6-BA were gradually increased, and reached to the peak values at 40 d. Culturing for 40 d, the contents of berberine in stems under 6-BA treatment were 10.74, 20.70, 22.68 and 19.38 mg/g DW, and enhanced by 5.13-, 9.83-, 10.87- and 9.15-fold compared with initial stage, and increased by 92.76%, 112.21% and 80.50% compared with CK at the same time (Fig. 4A). Culturing for 40 d, the contents of phellodendrine in stems treated by 10 mg/L and 30 mg/L 6-BA were enhanced by 0.71- and 0.42-fold, but 10 mg/L 6-BA reduced the content of phellodendrine compared with CK. Furthermore, the contents of phellodendrine under 6-BA treatment were all higher than in initial stage, and enhanced by 2.14-, 0.71- and 1.60-fold (Fig. 4B). The peak times of palmatine in stems of *Phellodendron chinense* seedlings under 6-BA treatment were appeared at 30 d and 40 d. Culturing for 40 d, the contents of palmatine in stems treated by 20 mg/L 6-BA was increased by 0.48-fold, but 10 mg/L and 30 mg/L 6-BA reduced its contents compared with CK (Fig. 4C).

NAA promoted the synthesis and accumulation of berberine in stems of *Phellodendron chinense* seedlings. With the prolongation of culture time, the contents of berberine in stems under different concentrations NAA were gradually increased, and reached to the peak at 30 d and 40 d. Culturing for 40 d, the contents of berberine treated by 10 mg/L, 20 mg/L and 30 mg/L NAA were 117.26, 111.77 and 81.82 percent of CK (Fig. 4D). The contents of phellodendrine in stems were reached to the peak values at 30 d and 40 d. Culturing for 40 d, 10 mg/L and 30 mg/L NAA promoted the accumulation of phellodendrine, and enhanced by 2.93-fold and 0.42-fold compared with CK, but 20 mg/L NAA deduced the content of phellodendrine, its content was only 49.67 percent of CK (Fig. 4E). The peak times of palmatine in stems of *Phellodendron chinense* seedlings under (0–30 mg/L) NAA treatment were appeared at 20 d and 40 d. However, NAA inhibited synthesis and accumulation of palmatine. Culturing for 40 d, the contents of palmatine in stems after NAA treatment were 64.67, 32.23 and 69.93 percent of CK (Fig. 4F). These results demonstrated that exogenous 6-BA and NAA regulate the synthesis and accumulation of medicinal ingredients in stems of *Phellodendron chinense* seedlings.

4. Discussion

Plant growth regulator played key roles in the processes of growth and development in plants. 6-Benzylaminopurine belong

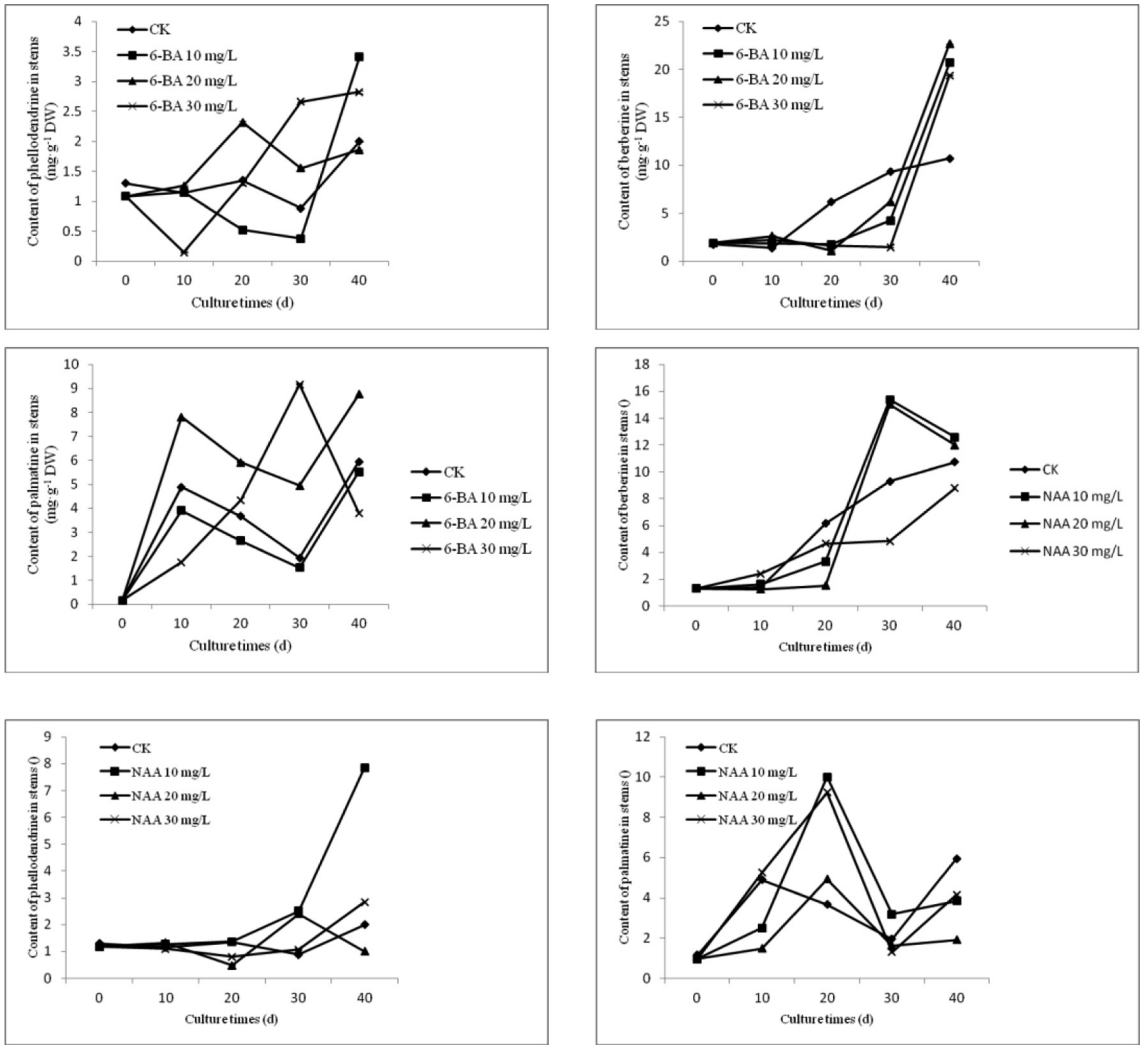

Fig. 4. Effects of exogenous 6-BA and NAA on the contents of medicinal composition in stems of *Phellodendron chinense* seedlings.

to cytokinin, and played significant physiological function in the courses of regeneration, production, quality, synthesis and accumulation of secondary metabolite and stress response (Chen and Yang, 2013; Pan et al., 2013; He and Shi, 2014; Singh et al., 2015). The previous study showed that proper concentration mepiquat chloride (DPC) could increase fresh weight of *scutllaria baicalensis* roots, enhanced the contents of baicalin and total flavonoids, but deduced the contents of baicalein and wogonin compared with CK (Hu et al., 2012). Compared with CK, a 3% sucrose medium added 3.0 mg/L 6-BA and 0.5 mg/L NAA enhanced the contents of curcumin and curcuminoids in imcrorhizomes of *curcuma aromatica* salisb which exposures to red light (Wu et al., 2015; Ong et al., 2017). Different concentrations of 6-BA or 6-BA and NAA combination significantly inhibited the growth of *Pueraria phasedoides* hairy roots, and reduced biomass and total content of isoflavone, but NAA inhibited the growth and accumulation of medicinal ingredient of *Polygonum multiflorum* hairy roots (Yu et al., 2006; He and Shi, 2014). So, exogenous 6-BA and NAA had key regulation in the processes of growth and accumulation of medicinal ingredients in plants, and had difference between different kinds of plants. In this paper, the results showed that different concentrations of 6-BA and NAA could obviously increase fresh weight and plant height of *Phellodendron chinense* seedlings compared with CK, especially 10 mg/L 6-BA and NAA (Fig. 1). 20 mg/L 6-BA and 30 mg/L 6-BA deduced the contents of chlorophyll in leaves of *Phellodendron chinense* seedlings, but 10 mg/L 6-BA enhanced the content of chlorophyll. Furthermore, 20 mg/L NAA deduced the content of phlorophyll, 10 mg/L and 30 mg/L NAA enhanced its contents, these results were consistent with the growth trend of *Phellodendron chinense* seedlings (Fig. 2). Culturing for 40 d, exogenous 20 mg/L 6-BA could obviously have enhanced the contents of berberine and palmatine in stems of *Phellodendron chinense* seedlings, and 10 mg/L 6-BA may significantly increase the content of phellodendrine compared with CK. 10 mg/L NAA enhanced the contents of berberine and phellodendrine, but different concentrations of NAA inhibited the synthesis and accumulation of palmatine compared with CK (Fig. 4). These results indicated that proper concentration 6-BA and NAA promote the growth and accumulation of medicinal ingredients of *Phellodendron chinense* seedlings.

During the process of metabolism, plants easily suffer external stresses, and will be produced large amount of reactive oxygen species (ROS) in the body. If ROS is not cleared in times, it will poison the growth and secondary metabolite. The plants scavenged ROS through antioxidants and antioxidants, and former are key scavenge system. SOD, POD and CAT are the major components of antioxidant system, and play key roles in the processes of growth and tress responses in plants (David, 2013; Mittler, 2016; Yang et al., 2017). Cytokinin 6-BA deduced the levels of SOD, POD and endogenous ethylene of cucumber hairy roots, 10 umol/L 6-BA enhanced the level of SOD, POD and CAT in detached leaves

of *Triticum aestivum*, and reduced the content of soluble protein (Shi et al., 2006; Huang et al., 2011). The yield and levels of SOD and POD activities of super hybrid rice (*Oryza sativa*) after spraying 30 mg/L 6-BA solution were increased (Pan et al., 2013). Compared with CK, 10 mg/L 6-BA and 20 mg/L 6-BA enhanced the levels of SOD, POD and CAT in leaves of *Phellodendron chinense* seedlings at 40 d. Culturing for 40 d, low concentrations NAA enhanced levels of SOD and CAT, but all concentrations of exogenous NAA decreased the level of POD compared with CK (Fig. 3). The enhancing of antioxidant activity levels of *Phellodendron chinense* seedlings, on the one hand, exogenous 6-BA and NAA stimulated and promoted the generation and accumulation of antioxidizes, on the other hand, high level of antioxidize activities could kept the ROS on the baseline level through degradation or transformation manner, protected normal growth of *Phellodendron chinense* seedlings.

5. Conclusion

Based on these results, we concluded that proper exogenous 6-BA and NAA had promotion on growth and synthesis and accumulation of medicinal ingredients of *Phellodendron chinense* seedlings. The synthesis of berberine, phellodendrine and palmatine was controlled by some genes, but the effects of exogenous 6-BA and NAA on the expression level and expression pattern were not cleared. Therefore, we will focus on the cloning, analysis of expression level and functional verification of these synthase genes in the future study.

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