Establishment of genetic transformation system of *Populus euphratica* and optimization of antibiotic concentration

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Abstract: Effects of hormones including NAA, IAA, 6-BA on leaf differentiation of *Populus euphratica* and antibiotics including Kanamycin (Kan), G418, Carbenicillin (Cab), Cefotaxime (Cef) on the growth and differentiation or rooting of various explants of *P. euphratica* were studied. Genetic transformation system mediated by *Agrobacterium* and optimum antibiotic concentrations for modified organism selection of *P. euphratica* were founded. Results shown that: the optimal culture medium for leaf differentiation of *P. euphratica* was MS+BA 0.5 mg/L+NAA 0.1～0.2 mg/L+sugar 25 g/L+agar5 g/L; at co-culture stage which leaves were used as explants, the optimum concentrations of Kan, G418, Cab and Cef were 10 mg/L, 7.5 mg/L, 200～600 mg/L, 200～400 mg/L respectively; at the stage of subculture or rooting of resistance buds, the optimum concentrations were 15～20 mg/L, 10～15 mg/L, 200～800 mg/L, 200～600 mg/L respectively.

Key words: *Populus euphratica*; genetic transformation system; antibiotic concentration; optimization

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Populus euphratica, having extremely strong stress resistance, is the only natural tall and big arbor species distributed in the front aridity and desert northwest area of China (Wang *et al.*, 1995; Wei, 1990). For physiological reasons, *P. euphratica* has the characteristic of few seeds, sexual reproduction handicap and rooting difficulty by cutting, which restrict its popularization and application seriously. Genetic engineering has the advantages of specific purpose, shorting breeding time and breaking hybridization limitation of different species so that it has became an important mean for genetic improving of *P. euphratica*. During genetic transformation, screening genes were linked often for the selection of genetic modified organism. Npt Ⅱ gene which coding neomycin phosphoric acid shift enzyme is a screening gene used most extensively in plant genetic engineering at present. It can phosphoric the amino glucosidal type antibiotic such as Kan (kanamycin), G418, Neo (neomycin), etc., thus enable genetic transformed cells has the ability to resist above antibiotics (Wang *et al.*, 1998). But researches indicated that antibiotics has also some inhibitions to plant tissues growth and differentiation (Guan *et al.*, 1994; Wang *et al.*, 1996). So the establishment of genetic transformation system and the optimization of antibiotic concentrations are the necessary precondition before genetic transformation of *P. euphratica*. This paper focused on and studied the effects of 6-BA, IAA and NAA on leaf and callus differentiation of *P. euphratica* and Kan, G418, Cab and Cef on the growth and differentiation or rooting ability of various explants of *P. euphratica*. Results of this research selected the optimal culture medium for leaf differentiation, screened and optimized the kinds and concentration of antibiotics, which established the foundation for the genetic transformation of *P. euphratica* with *Agrobacterium tumefaciens*.

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1 Materials and Methods

1.1 Materials

Test-tube plantlets of *P. euphratica* for test were from the key laboratory of genetics and breeding in forest trees and ornament plants (Beijing Forestry University), Ministry of Education. Leaf and various explants for test were selected from test-tube plantlets cultured after 20～30 d (Zhang *et al.*, 2001; Gu *et al.*, 1999; Ding *et al.*, 2003). The *A. tumefaciens* of LBA4404 saved in our lab. Kan and G418 made in Japan. Other antibiotics brought from Dingguo corp.

1.2 Culture media

Aseptic seedlings come from adventitious buds of *P. euphratica* cultured in vessels. The leaves were selected from top to bottom of No. 1 to 3 and the obverse side of leaves laid upwards to the following culture media of number 1 to 8. Every group contained 6 explants and repeated 4 times.

The antibiotics were added to the sterilized culture medium when the temperature of medium reduces to under 50 degree and mixed well before solidify.

1.3 Effects of antibiotics on the growth, differentiation and rooting of explants

The explants of leaf, stem and virus-free seedlings were planted in culture medium containing different kinds and concentrations of antibiotics and then their growth situations were observed. Investigation and statistics of index carried on 30 days later. Concentration of Kan and G418 were divided into 0, 2. 5, 5, 7. 5, 10, 15 mg/L group and Cab and Cef were divided into 0、200、400、600、800 mg/L group also. Every concentration group included 5 culture vessels which planted 5 pieces (clones) under 28 ℃ and repeated 3 times, 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of illumination intensity, 12～14 h/d for illumination.

2 Results and Analysis

2.1 Effects of different hormone concentration group on leaf regeneration of *P. euphratica*

Conclusions from table 1 showed that: on the culture media with NAA, at the concentration range of BA from 0. 5 to 1. 0 mg/L, the leaf regeneration efficiency of all the groups reached up to 100%. Whereas on the culture media with IAA, at the concentration range of BA from 0. 1 to 1. 5 mg/L, the highest leaf regeneration efficiency reached up to 83. 3% and each adventitious bud of leaf only 5. 7 at most on average. Within the specific limits, the quantity of the adventitious bud of leaf relates to BA and NAA (IAA) ratio. When the ratio was 5, regeneration frequency and quantity of adventitious bud of leaf on average reached the maximum level. Among them, the culture media containing antibiotic concentration of BA 0. 5 mg/L and NAA 0. 1 to 0. 2 mg/L were relatively favorable to the leaf regeneration. Variance analysis and multiple comparing to the quantity of adventitious bud indicated that the culture medium numbers of 2 and 4 were the best. Therefore, the best culture medium prescription for leaf regeneration of *P. euphratica was*: MS+BA 0. 5 mg/L+NAA 0. 1～0. 2 mg/L+sugar 25 g/L+agar 5 g/L.

Table 1　Effects of different BA, NAA and IAA level on adventitious bud regeneration

No.	Culture medium ($mg \cdot L^{-1}$)	No. of leaf	Leaf number of adventitious bud	Regeneration frequency (%)	Mean number of regeneration bud
1	BA 0. 1+IAA 0. 1	24	9	37. 5	1. 4 c
2	BA 0. 5+NAA 0. 1	24	24	100	12. 8 a
3	BA 0. 5+IAA 0. 1	24	20	83. 3	2. 6 c
4	BA 0. 5+NAA 0. 2	24	24	100	10. 8 a
5	BA 0. 5+IAA 0. 2	24	18	75	5. 7 c
6	BA 1. 0+NAA 0. 1	24	24	100	8. 6 b
7	BA 1. 0+IAA 0. 1	24	9	37. 7	4. 6 c
8	BA 1. 5+IAA 0. 1	24	15	62. 5	1. 3 c

Note: The small letter means significant difference at 0. 05 level. The same below.

2.2 Effects of Kan on leaf regeneration, stem differentiation and rooting of *P. euphratica*

Results from the Fig. 1 showed that all the culture media containing Kan had inhibition to the regeneration and differentiation or rooting of *P. euphratica* explants. With the rising of Kan concentration, the regeneration and differentiation frequency of leaf and stem were reduced gradually. Kan had more influences to the regeneration and differentiation frequency of explants. When the concentration of Kan was at 2. 5 mg/

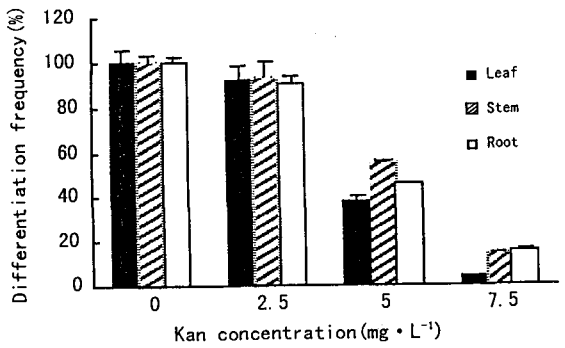

Fig. 1 Effects of kanamycin on morphogenesis of *Populus euphratica* in tissue culture

L, the regeneration and differentiation frequency of leaf and stem reached to more than 90%; when the concentration of Kan equaled to 5 mg/L, it reduced to 38% from 100% of contrast, stem differentiation reduced to 56% rapidly too; as the concentration of Kan was more than 7.5 mg/L, the leaf regeneration for adventitious bud were totally suppressed. The inhibition of Kan still behaved as organization albino and differentiation delaying. Higher Kan concentrations accompanied with higher rate of albino seedlings. When Kan concentration equaled to 2 mg/L, only a small amount of albino seedlings were produced; when Kan concentrations were more than 5.0 mg/L, the color of adventitious bud or axillary bud appeared yellowish-white, and only appeared leaf extend without growth point. When culture continued, buds stop growing and become albino gradually. In the culture of adding Kan, the acquisition time of the bud was postponed 5 days than contrast. It was obvious that Kan had strong inhibition on the regeneration and differentiation of leaf and stem.

The rooting ability of *P. euphratica* was very sensitiveness to Kan too. It was found that explants which grew in culture media including no kan, shoot and root wound can produce phenomenon of expanding after 10 d, and had production of adventitious root quickly which can reach 3 cm finally, and there was more figure. When Kan concentration was at 5.0 mg/L, it can suppress virus-free seedlings of *P. euphratica* to take root obviously. Though some shoots can produce adventitious root too, the root was relatively short, shorter than 1 cm, root quantity less, and plant high grow suppressed; when the concentration of Kan equaling to 7.5 mg/L, the root produced was shorter, the suppressed degree of high growth of the plant increases; when Kan concentration was more than 10 mg/L, no taking root phenomenon appeared, the plant was short and small and the top leaf yellowy seriously. So, stem section of *P. euphratica* can not take root in screening culture which Kan concentration is greater than 10 mg/L. Only the adventitious bud transformed through genetics engineering may take root, that was the measure to select the transgenic adventitious bud or not.

2.3 Effects of G418 on leaf regeneration, stem differentiation and aseptic seedlings rooting of *P. euphratica*

Fig. 2 showed that the inhibition of G418 on regeneration and differentiation or rooting of *P. euphratica* explants were more obvious than Kan. With the rising of concentration, the regeneration and differentiation frequency of leaf and stem, the taking root rate of shoot were reduced rapidly. In the treatment of adding G418 with 5 mg/L, the blade loses green by a large scale, only split up a small amount of adventitious bud in the petiole and blade notch place. The regeneration frequency of the blade was dropped to 18% and the induce frequency rooting and division of stem to reduce by a large margin, lower to 15% and 25% separately; as G418 concentration equaled to 7.5 mg/L, adventitious bud regeneration, stem differentiation and root inducing inhibited totally and no blade and petiole growing phenomenon appeared. The whole blade loses green and not differentiation of the adventitious bud. There was no adventitious bud to split up even if lengthen culture time, and then the blade withered. The apical dominance and induced rooting of virus-free seedlings were suppressed seriously. The plants appeared short, small, yellow top blade and out of shape to twist. So 7.5 mg/L can be as the critical concentration of G418 that screening the transgenic virus-free seedlings of *P. euphratica*.

2.4 Effects of Cef on leaf regeneration, stem differentiation and aseptic seedlings rooting

The experimental results indicated: Cef had little effects on root inducing of aseptic seedlings of *P. euphratica*; the root inducing rate of all groups were above 80% at different concentration level; when the

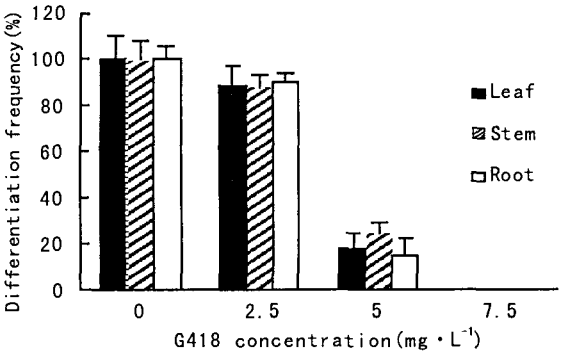

Fig. 2 Effects of G418 on morphogenesis of *Populus euphratica* in tissue culture

concentrations were lower than 400 mg/L, the differentiation of the blade and stem section was influenced little also; but with the rising of the concentration, the division of the blade and stem section were suppressed, more inhibition to the section of the stem; when Cef concentration equals to 800 mg/L, the rate of blade and stem regeneration were dropped to under 80% and 60% respectively; when Cef concentration was more than 800 mg/L, adventitious bud that form wound blade was no obvious extending and growing with culture time extension, which prove the function of Cef on adventitious bud mainly to suppress the extending and growing. When Cef concentration exceeds 800 mg/L, the production time of the blade adventitious bud postpones 2 to 3 days more than contrast and the root and division time of stem postpone 3 to 5 days.

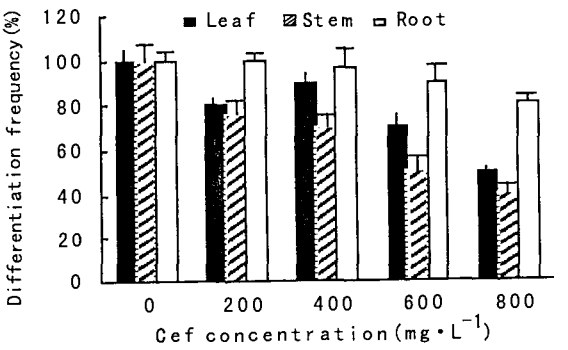

Fig. 3 Effects of Cef on morphogenesis of *Populus euphratica* in tissue culture

2.5 Effects of Cab on leaf regeneration, stem differentiation and aseptic seedlings rooting

Compared with Cef, carboxyl benzyl penicillin had less influence on leaf regeneration, stem differentiation and aseptic seedlings rooting. Acting as the concentration of benzyl penicillin of carboxyl from 0 to 1 000 mg/L, all the culture media can form adventitious bud, auxiliary bud, root, and the division frequency of adventitious bud and root were comparatively steady. The division frequency of adventitious bud, axillary bud and the rooting frequency of aseptic seedlings were higher than corresponding concentration of Cef generally. When the Cab concentration equaled to 600 mg/L, leaf regeneration and stem differentiation rate were 91%, 76% respectively, aseptic seedlings rooting rate was 100%. New blade grown fast than that of Cef treated usually and all blades kept green; when the Cab concentration was at 600 to 800 mg/L, the blade begins to taken off green gradually; when the Cab concentration was at 1 000 mg/L, leaf regeneration and stem root inducing frequency were all above 90%, regeneration bud displayed slight glass symptom.

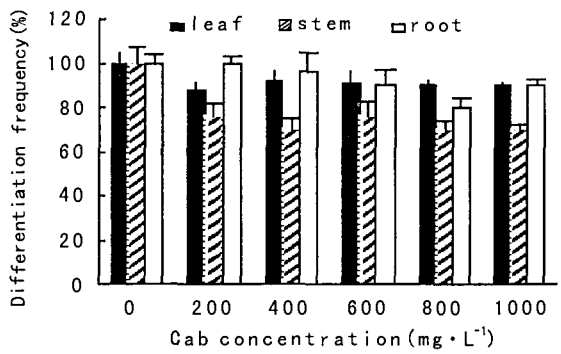

Fig. 4 Effects of Cab on morphogenesis of *Populus euphratica* in tissue culture

3 Conclusions and Discussions

While the plant genetic transformation, nptⅡ usually regarded as the screening mark gene, the expression result of this gene give the resisting of transgenic plant to kanamycin or G418. Understanding the experimental result of this research will help to adopt the proper choice pressure in plant transgenic studies and control the quantity of no-transforming body under the minimum limit. Kan and G418 have strong inhibition on the leaf regeneration, stem differentiation and aseptic seedlings rooting of *P. euphratica*. The inhibition of G418 was more remarkable. Culture medium con-

taining Kan concentration of 10 mg/L or G418 concentration of 7.5 mg/L, can suppress leaf differentiation of *P. euphratica* totally and can't emerge adventitious bud, axillary bud or adventitious root. So, Kan or G418 were relatively suitable for and regarded as the screening antibiotic. In the course of choosing, in order to prevent more none transgenic buds inducing, its concentration can be raised properly to Kan 15～20 mg/L or G418 10～15 mg/L. However, even use higher Kan or G418 concentration, there are specific plants to escape antibiotic selection and survive. So, as the receptor of gene transformation, there also exist the phenomenon of not transforming adventitious bud after screen of antibiotic pressure.

Cef and Cab were the bactericide which used extensively during plant genetic transformation mediated by agricultural bacillus (Zhang *et al.*, 2000; Lin *et al.*, 1995). But both Cef and Cab have different impacts on different plant tissues. In this experiment, Cef and Cab were relatively less inhibition on leaf regeneration, stem differentiation and aseptic seedlings rooting of *P. euphratica*. But it can suppress the blade regeneration and stem section to split up under high concentration and can suppress the excessive reproduction of the agricultural bacillus completely. Confirm tentatively from above that the suitable concentration of antibiotic at every stage during genetic transformation of *P. euphratica* were: during transgenic buds selection stage of blade (or stem section) the suitable concentration of Cef or Cab were the range of 200 to 400 mg/L; during rooting selection stage of the transgenic aseptic seedlings the suitable concentration of Cef or Cab were the range of 200 to 600 mg/L.

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胡杨遗传转化体系的建立及抗生素浓度的优化

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摘 要：探讨了不同激素浓度对胡杨叶片分化以及卡那霉素、G418、羧苄青霉素和头孢霉素4种抗生素对胡杨不同培养阶段外植体生长、分化或生根的影响，确定了由农杆菌介导的胡杨遗传转化研究中抗生素种类和转化体的筛选浓度，建立了适于胡杨叶片转化的遗传转化体系。结果表明：胡杨叶片再生的最佳培养基为MS+BA 0.5 mg/L+NAA 0.1～0.2 mg/L+白砂糖25 g/L+琼脂5 g/L；在叶片转化筛选阶段，卡那霉素和G418的适宜浓度分别为10 mg/L和7.5 mg/L，羧苄青霉素和头孢霉素的适宜浓度为200～600 mg/L和200～400 mg/L；在抗性芽生根培养时，卡那霉素和G418分别为15～20 mg/L和10～15 mg/L，羧苄青霉素和头孢霉素为200～800 mg/L和200～600 mg/L。

关键词：胡杨；遗传转化体系；抗生素浓度；优化