For. Stud. China, 2007, 9(4): 279–282
DOI 10.1007/s11632-007-0044-9

RESEARCH ARTICLE

Contamination and browning in tissue culture of *Platanus occidentalis* L.

Tao Feng-jie *Zhang Zhi-yi*[*] *Zhou Jun* *Yao Na* *Wang Dong-mei*

Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, Beijing Forestry University, 100083, P. R. China

Abstract Twigs of 2–3-year-old *Platanus occidentalis* L. were used as experimental material to find the causes for the contamination and browning in the initial stages of tissue cultures. To compare the degree of browning of explants picked off from different growing seasons, the experimental material was excised from trees on each of the first ten days in January, March, May and July, 2006. The results indicated that the contamination and browning rates of the material cut off in January (14.2% and 30.6%, respectively) and March were somewhat lower than those in July. The pretreatment of soaking the explants in different anti-oxidants and absorbents at the same time could diminish some side effects. The pretreatment of using 10 $g \cdot L^{-1}$ vitamin C reduced the contamination and browning rate effectively. An orthogonal experiment showed that the optimal factor and level arrangement is 0.5 $mg \cdot L^{-1}$ BA, 2.0 $g \cdot L^{-1}$ active carbon and 1.5 $g \cdot L^{-1}$ PVP which resulted in a browning rate of only 16.5%. In general, sampling period, physical properties and pretreatment of explants are the main factors responsible for the contamination and browning of material in the initial stages of *P. occidentalis* tissue cultures.

Key words *Platanus occidentalis* L., tissue culture, contamination, browning

1 Introduction

Platanus occidentalis L., usually called the American phoenix tree, is one of the more important woody plants in the Platanaceae family. *P. occidentalis* has strong adaptability that permits it to grow widely in China. As well, this tree can reduce noise levels and prevent dust in cities. Moreover, this tree species can be pruned over a considerable period of time, is easily sculpted and propagated as well (Guo, 2004). Because of these strong properties, *P. occidentalis* is entitled to be called "the king of avenue trees" and favored by many horticultural enthusiasts. The sprouts, twigs and fruits of *P. occidentalis* are covered by floss which is spread everywhere by the wind when they are mature, especially from trees that are bearing fruit. Between April and June of every year, the floss and pollen fall to the ground while many old dried fruits on the trees open up. What is worse, this floss and pollen, blown into the air in the form of small sheets, do not only pollute the air but can also cause serious respiratory allergic reactions (Liu et al., 2001).

Over the past 20 years, Chinese scientists have persisted in the struggle for cultivating new varieties of this species with less fruit or no fruit at all by artificial mutation and to reduce the fruit set of existing trees of *P. occidentalis* by crown grafting, impairing fruits by pruning and chemical restraints (Cao, 2000, Zhang, 2000), but the effects of those efforts have been disappointing. With today's rapid advances in cell and genetic engineering, scientists are striving to find new approaches, for instance with genetic engineering, to achieve the goal to grow *P. occidentalis* with less floss and less fruit, instead of with traditional breeding methods. A major obstacle is encountered in the process of establishing a *P. occidentalis* regeneration system while the system is indispensable for *P. occidentalis* bioengineering. This major obstacle is the fact that twigs, cultured on a medium, always become brown in the initial culture. This browning of twigs prevents further culture of twig callus. Therefore it is necessary to explore a suitable and synthetic cultural condition for the cultivation of twigs.

2 Materials and methods

2.1 Material

Twigs of 2–3 years old *P. occidentalis* from an experimental nursery in Hebei were used as the source of explants.

2.2 Methods

2.2.1 Pretreatment of material

Initially, the buds were soaked in a solution of 80% (v:v) commercial bleach for about 20 min. Then, the

[*]Author for correspondence. E-mail: zhangzy@bjfu.edu.cn

material was washed by flowing water for one hour. After that, the experimental material was transferred to a sterile hood, where their surfaces were disinfected for 30 s using 75% ethanol and then dipped in a solution of 0.1% $HgCl_2$ for 4–8 min and rinsed out four times with sterile water. Finally, the twigs were inoculated in MS media supplemented with 30 $g \cdot L^{-1}$ saccharose and 6 $g \cdot L^{-1}$ agar and their pH regulated from 5.7 to 5.8. The temperature was kept at 25 ± 1°C. Per day 13 hours of illumination was maintained indoors (generally from 08:00 to 21:00) with a light intensity of 2,000 lx. The basic tissue culture medium was MS.

2.2.2 Effect of growing season on browning

In order to compare the browning effect on explants during different growing seasons, the experimental material was collected on each of the first ten days in January, March, May and July in 2006.

2.2.3 Browning effect of anti-oxidant and absorbent pretreatment

The branches were initially cultivated in the green house for several days. Then the explants, excised from the branches, were soaked with various anti-oxidants and absorbents (VC, PVP, AC) as a pretreatment. The concentration of VC was 0.1–1.0 $g \cdot L^{-1}$, that of PVP 0.5–2.0 $g \cdot L^{-1}$ and AC 1.0–4.0 $g \cdot L^{-1}$.

2.2.4 Screening of optimal arrangement

An orthogonal test $L_9(3^4)$ (Gai, 2000) was employed to find the optimal arrangement of three factors prescribed as the concentration of BA (factor A), AC (factor B) and PVP (factor C). Each factor consisted of 3 levels. The experimental design of factors and levels are shown in Table 1.

Table 1 Experimental design of orthogonal test $L_9(3^4)$

Level	Factors			
	Concentration of BA (A) ($mg \cdot L^{-1}$)	Concentration of AC (B) ($g \cdot L^{-1}$)	Error variance control (D)	Concentration of PVP (C) ($g \cdot L^{-1}$)
1	0.5	1.0		1.0
2	1.5	1.5		1.5
3	2.5	2.0		2.0

Table 2 Impact of sampling period on the contamination and browning rates

Sampling period	Sterilization time (min)	Contamination rate (%)	Browning rate (%)
January	8	14.2	30.6
March	8	20.3	43.5
May	8	35.8	50.4
July	8	47.5	68.7

3 Results

3.1 Impact of sampling period on contamination and browning effect ratios

The contamination and browning rates of the material cut off in January and March were smaller than those in July while the sterilization times were the same. The rates in January were the smallest among all months. The results are shown in Table 2.

3.2 Effect of material pretreatment on browning

The disinfected explants were soaked with anti-oxidants and absorbents as shown in Table 3. As the concentration of VC increased, the browning fractions became lighter. When the concentration was 10 $g \cdot L^{-1}$, the browning fractions faded quickly. Meanwhile, changing the concentrations of PVP, VC and soaking period did not ease the browning rates, but the time in which the cultured material became darkened was, to a certain extent, prolonged. We strongly recommend the VC pretreatment. While the explants were incised in anti-oxidants or absorbents, or fresh tissue material was saved with these anti-oxidants or absorbents, the pretreatment was able to segregate material, on which we operated, from oxygen and repress the PPO activity of the fresh fractions, so that the browning rate decreased.

3.3 Results and analysis of orthogonal test

Table 4 shows the browning ratios of the different arrangements of factors and levels. The browning rates were transformed by an inverse transformation. The browning rate of $A_1B_3C_3$ was the smallest where the concentration of BA was 0.5 $mg \cdot L^{-1}$, that of AC 2.0 $g \cdot L^{-1}$ and PVP 2.0 $g \cdot L^{-1}$.

In Table 5 *R* is the range among the results of different levels of each factor. Given the mean value (*X*) of the three factors respectively, the optimum combination of factors was $A_1B_3C_2$ which minimized the browning effect. However, an analysis of variance and multiple comparisons still needed to be done in order to verify the significance of the levels of the factors A, B and C in our results.

3.3.1 Analysis of variance

The analysis of variance is shown in Table 6. Our experimental results show that the effect of the factors A, B and C are significant. That means that some concentrations of BA, AC and PVP can significantly affect the problem of browning of *P. occidentalis*.

Table 3 Material pretreatment vs. browning effects

Additive category	Concentration ($g \cdot L^{-1}$)	Growth evaluation after pretreatment	Browning effect descriptions
VC	0.1–1.0	Slow growth or even cessation of growth	Browning rate did not change with pretreatment time
	1.0–10	Postpone browning for about 8 days	Browning fraction faded quickly with increased density
PVP	0.5–1.0	Slow growth	No clear change after pretreatment
	1.0–2.0	Defer browning for about 10 days	
AC	1.0–2.0	Good growth during anaphase	No clear change
	2.0–4.0	Slow growth during anaphase	

Table 4 Results of orthogonal test $L_9(3^4)$

No.	Factor combination	Explants of inoculations	Browning explants	Browning rate (%)	arcsin $\sqrt{x}$
1	$A_1B_1C_1$	25	15	60.00	50.77
2	$A_1B_2C_2$	20	7	35.00	36.27
3	$A_1B_3C_3$	23	6	26.09	30.72
4	$A_2B_1C_2$	20	13	65.00	53.73
5	$A_2B_2C_3$	18	10	55.56	48.22
6	$A_2B_3C_1$	17	8	47.06	43.34
7	$A_3B_1C_3$	19	18	94.74	76.69
8	$A_3B_2C_1$	21	16	76.19	60.80
9	$A_3B_3C_2$	23	11	47.82	43.74

Table 5 Analysis concerning the arcsin $\sqrt{x}$ transformation of $L_9(3^4)$ test results

Mean value (X)	Factors		
	A	B	C
X_1	40.36	73.25	61.08
X_2	55.87	55.58	49.27
X_3	72.91	40.32	58.80
R	32.55	32.92	11.81

Table 6 Analysis of variance of the arcsin $\sqrt{x}$ transformed data of test $L_9(3^4)$

Source of variation	*df*	*SS*	*MS*	*F*	$F_{0.01}$
A	2	4,772.264	2,386.132	684.04**	99.4
B	2	4,886.420	2,443.210	700.40**	
C	2	706.196	353.098	101.22**	
Error variance	10	34.882	3.488		
Total	16	10,559.762			

** significant at $\alpha = 0.01$

3.3.2 Multiple comparisons of the results

Multiple comparisons among the factors A, B and C are listed in Table 7. Factor A was composed of A_1, A_2 and A_3. The differences between the levels are significant. A_1 is the optimal level which repressed browning most heavily. For the factors B and C, B_3 is the optimal level among the different levels of factor B and C_2 the optimal level of factor C (Table 5). Consequently, $A_1B_3C_2$ is the optimal level arrangement among all the levels of arrangements considered. In order to ascertain whether the $A_1B_3C_2$ is indeed the optimal level among all the levels of arrangements, we repeated our experiment with an addition of combination $A_1B_3C_2$. The browning rate of $A_1B_3C_2$ was only 16.5%, which definitely confirmed our prediction.

4 Discussions

In our present study, we were dealing with aseptic twigs for tissue culture. At the initial stage of tissue culture, the tissues atrophied and gradually perished. During the experiment, we did not observe any evidence of exogenous substances that could make the tissues brown and spread into the cultures. Thus the reason for the damage inflicted might be that the medium and temperature are not suitable for the explants.

Xu et al. (1997) and Li and Qiao (2001) reported that browning can be caused by phenolic compounds at the chemical level. The phenolic compounds could activate polyphenoloxidase (PPO) of explants and change the metabolism of tissue cells and form brown quinine substances through its oxidation (Chen, 2004; Chen et al., 2005). The brown quinine substance will gradually enter the tissues cultured on the medium and further repress the activities of other enzymes and as a result, poison other contents of the medium. However, the anti-oxidants could repress the oxidation of the phenolic compounds by changing the oxidation potential of phenolic compounds around the explants and consequently alleviate the browning instead.

Generally, the pretreatment, anti-oxidants and absorbents can effectively control the browning. Anti-oxidants can prevent the oxidization of phenols and absorbents can adsorb quinines. Anti-oxidants include MC, 8-hydroxyquinoline, sulfate, argent, nitras (Zhong et al., 2002), VC, cysteine, globulariacitrin,

Table 7 Multiple comparisons of the A, B and C factors at different levels

A factor (BA)			B factor (AC)			C factor (PVP)		
Level	X_i-40.36	X_i-55.87	Level	X_i-40.32	X_i-55.58	Level	X_i-49.27	X_i-58.80
X_3	32.56**	17.05**	X_1	32.93**	17.67**	X_1	11.81**	2.28
X_2	15.51**		X_2	15.26**		X_3	9.53*	
X_1			X_3			X_2		

Note: ** extremely significant ($P < 0.01$), * significant ($P < 0.05$).

ovalbumin and so on. Absorbents consist of AC and PVP (Han et al., 1995; Park et al., 2000; Chen, 2004). The explants of Shanxi walnut were pretreated with 20% sodium hyposulfite to decrease the browning rate. Of various pretreatments of Shanxi walnut, it was found that the effects of VC would last only for a short time, the effects of PVP remained over a longer period and AC makes the plantlets bloom, expedites root growth and enhances the absorption of nutrients. In addition, active carbon has some side effects in counteracting the browning: it absorbs growth regulators because of its uptake of poisonous phenolic compounds at the same time. Thus, the density of active carbon should be carefully reduced. Besides, adding anti-oxidants and absorbents during culture could depress the browning effectively. Many experimental results indicate that 2 $g \cdot L^{-1}$AC and 1.5 $g \cdot L^{-1}$ PVP is an optimum level (Park et al., 2000; Qin and Sun, 2002; Zhou et al., 2002).

The density of BA could affect the growth and browning of the twigs. Browning and differentiating rate increased and browning time was moved up as the density of BA increased, the plants grew slowly and turned brown and dark. The lower density of BA could accelerate the synthesis of phenolics (Yao et al., 1999) and that of the polyphenol oxidase (Qiu and Cao, 1989). The same phenomenon also occurred in walnuts (Zhang et al., 2003) and potatoes (Zhang et al., 2004).

Basically, we found in our experiments that physical properties and collection time were the main causes for browning. It is necessary for us to probe into the mechanisms at physical, biochemical and genetic levels in order to solve further the problem of browning in tissue cultures.

Acknowledgement

This study was supported by the Beijing Forestry University Master Candidate Independent Research Fund (05jj037).

References

Cao A P. 2000. Applying of fruitless *Platanus occidentalis* Linn. in city virescence. Chin. Hort. J., 4: 76 (in Chinese with an English abstract)

Chen F, Li L, Gong W. 2005. Discussion of browning effects during the tissue cultural of plant. Northern Hort. J., 2: 69 (in Chinese with an English abstract)

Chen K. 2004. Browning mechanism and depress method during the tissue culture. J. Anhui Agric. Sci., 32(5): 1,034–1,036 (in Chinese with an English abstract)

Gai J Y. 2000. Experimental Statistical Method. Beijing: China Agricultural Press, 382 (in Chinese)

Guo C P. 2004. Horticulture virescence trees *Platanus*. West For. Sci. Tech., 35–36 (in Chinese with an English abstract)

Han S Y, Qi L W, Zhang S G. 1995. Study on browning effect of walnut tissue culture. J. Shanxi Agric. Univ., 4: 339–341 (in Chinese with an English abstract)

Li H X, Qiao N J. 2001. Study on decreasing the browning of explants of Cangxi pear. J. Southwest Agric. Univ., 23(6): 524–526 (in Chinese with an English abstract)

Liu H Y, Fu S L, Gao D Y. 2001. Renovation and protection of the middle and old choiceness *Platanus occidentalism* Linn. J. Hebei For. Tech., 1: 1–5 (in Chinese with an English abstract)

Park Y S, Murthy H N, Paek K Y. 2000. Mass multiplication of protocorm like bodies using bioreactor system and subsequent plantre generation in *Phalaenopsis*. Plant Cell Tiss. Organ Cult., 63: 67–72

Qin H L, Sun H M. 2002. Review of butterfly orchid. J. Henan Profess. Tech. Teach. Coll., 30(2): 31–35 (in Chinese with an English abstract)

Qiu W D, Cao Z Y. 1989. Applied Technology of Plant Tissue Culture. Beijing: Higher Education Press, 96–98 (in Chinese)

Xu Z B, Fu Z S, Yuan Y P. 1997. The browning phenomenon of the plant tissue culture. J. For. Agron.-Crop., 1: 55–56 (in Chinese with an English abstract)

Yao H J, Luo X F, Tian Y T. 1999. Development of explants browning researches. J. Beijing For. Univ., 21(3): 78–84 (in Chinese with an English abstract)

Zhang L M. 2000. Prune and fruits control technology on the *Platanus occidentalis* Linn. J. Jiangsu For. Sci. Tech., 27(3): 37–38 (in Chinese with an English abstract)

Zhang S H, Wang D, Wang Q. 2004. The browning elements regarding to *Phytophthora infestan* and the functions of $AgNO_3$ on the browning effects and cells division. J. Chin. Phytoph., 18(2): 77–81 (in Chinese with an English abstract)

Zhang W F, Gao J S, Ou Y H, Yang P W. 2003. Primary study on browning control in tissue culture of pellicular walnut. Decid. Fruits, 3: 4–7 (in Chinese with an English abstract)

Zhong M Q, Lou C F, Tan J Z. 2002. Function of $AgNO_3$ during mulberry genetic translation. J. Trop. Subtrop. Plant, 10(1): 74–76 (in Chinese with an English abstract)

Zhou J H, Ye C H, Chen X G. 2002. Study on corm proliferation culture of butterfly orchid. J. Zhongkai Agric. Tech. Coll., 15(3): 13–17 (in Chinese with an English abstract)

(Received December 12, 2006 Accepted June 23, 2007)