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Effects of Several Antioxidants on Browning Inhibition in *in vitro* Culture of Leaves Harvested from Adult Plants in Loquat (*Eriobotrya japonica* L.)

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Abstract: The attempt in this study was to find efficient methods of overcoming explant browning in leaf culture in which the leaf explants were harvested from adult plants in loquat (*Eriobotrya japonica* L.). Antioxidants and adsorbents including $Na_2S_2O_3$, Polyvinyl Pyrrolidone (PVP), $AgNO_3$, Activated Charcoal (AC), citric acid, Vitamin c (Vc) and bleaching powder were employed to study their effects on browning control by immersing explants and adding to the medium. The results indicated that 0.5 mg L^{-1} $AgNO_3$ added to the medium showed the best effect on browning control while all the other measures did not have obvious effects.

Key words: Browning, leaf culture, loquat (*Eriobotrya japonica* L.) , adsorbents, plants, China

INTRODUCTION

Loquat (*Eriobotrya japonica* L.) as an important economic fruit crop is widely cultivated between 20 and 35° latitude in both Northern and Southern hemisphere (Badenes *et al*., 2000; Vilanova *et al*., 2001). This crop is characterized by high level of heterozygosity, long reproductive cycle and juvenile phase which makes it difficult and time-consuming to use conventional breeding approaches. Genetic transformation has shown to be a powerful strategy for cultivar improvement while maintaining its excellent integrity by adding new traits and may effectively serve the genetic improvement in loquat. Plant regeneration via *in vitro* culture of leaves from adult mother plants is a prerequisite for practical cultivar improvement through agrobacterium-mediated genetic transformation. Some investigations have been conducted in loquat tissue culture including shoot-tip culture (Yang *et al*., 1983), embryo culture (Qie and Zhang, 2009), endosperm culture (Zhuang *et al*., 1982), anther culture (Li *et al*., 2008) and protoplast culture (Lin *et al*., 1995; Lin and Chen, 1996). Nevertheless, no reliable plant regeneration technique has been available for leaf culture of adult plants, although Wu *et al*. (2007) realized adventitious shoot regeneration from mature cotyledons and leaves of seed-derived seedlings. Consequently, there is a need to establish a high frequency regeneration system via leaf culture using adult mother plant explants which will greatly improve the breeding efficiency in loquat.

Explant browning constitutes the first barrier in *in vitro* culture of leaves harvested from adult mother plants in loquat. Till now, no report can be found about browning inhibition in loquat. This study for the 1st time was designed to systematicaly study the effects of different treatments on browning inhibition and callus induction using leaf explants harvested from adult mother plants in loquat.

MATERIALS AND METHODS

Young leaves of loquat (*Eriobotrya japonica* L.) cv. Dawuxing were used for the experimental materials harvested from 10 years old mother plants in Biotechnology Research Center for Horticultural Crops in Sichuan Agricultural University, China.

The leaves sampled in the experimental plots were rinsed for 1 h with the running water. During the process of rinsing, the dirts on the leaves' surfaces were brushed off with a soft toothbrush. Then the leaves were immersed in washing powder water for 3-5 min for further surface-cleaning followed by another rinse for 1 h. The final step of sterilization and further operations were carried out in a laminar air-flow cabinet under aseptic conditions. The leaves were surface-sterilized by immersing in 75% (v/v) alcohol for 20 sec and 0.1% (w/v) mercuric chloride solution ($HgCl_2$) for 8 min with periodic agitation and washed with sterile-distilled water for 5 times. Finally, the leaf explants (0.5×0.5 cm) were plated in the conical flasks (100 mL) containing 25 mL of medium. $AgNO_3$ (0.05, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}), citric acid (150 mg L^{-1}), Vc (150 mg L^{-1}), bleaching powder (5%), $Na_2S_2O_3$ (0.05, 0.1, 0.3, 0.5 and1.0 mg L^{-1}), AC (0.05, 0.1, 0.3, 0.5 and 1.0 mg L^{-1}) and PVP (1, 2, 3, 4 and 5 mg L^{-1})

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were used at different concentrations. The explants were treated with the antioxidants or adsorbents for different immersion time. Light and dark treatment were used in the culture (Table 1-6).

Table 1: Effects of immersing explants with bleaching powder, citric acid and Vc

	Bleaching powder		Citric acid		Vc	
Time (min)	Light	Dark	Light	Dark	Light	Dark
10	0.60^{ab}	0.68^{bc}	0.77^{a}	0.72^{a}	0.58^{a}	0.53^{a}
20	0.55^{a}	0.62^{b}	0.72^{a}	0.80^{ab}	0.65^{b}	0.63^{b}
40	0.57^{a}	0.53^{a}	0.75^{a}	0.78^{ab}	0.68^{bc}	0.62^{b}
60	0.68^{b}	0.70^{bc}	0.82^{ab}	0.85^{b}	0.68^{bc}	0.68^{c}
90	0.82^{c}	0.83^{c}	0.83^{ab}	0.82^{ab}	0.80^{c}	0.84^{c}

Table 2: Effects of PVP added to the medium on browning inhibition in loquat leaf culture

	No. of browning		Browning rates (%)	
Concentration ($g\ L^{-1}$)	Light	Dark	Light	Dark
1	51	48	85.0	80.0
2	47	43	78.3	71.7
3	39	41	65.0	68.3
4	45	45	75.0	75.0
5	43	48	71.7	80.0

Table 3: Effects of $AgNO_3$ added to the medium on browning control in loquat leaf culture

Concentration ($mg\ L^{-1}$)	No. of browning	Browning rate (%)
0.05	27	45.0
0.1	22	36.7
0.3	14	23.3
0.5	27	28.3
1.0	23	38.3

Table 4: Effects of AC added to the medium on browning control in loquat leaf culture

	No. of browning		Browning rates (%)	
Concentration ($mg\ L^{-1}$)	Light	Dark	Light	Dark
0.05	56	49	93.3	81.7
0.1	48	51	80.0	85.0
0.3	52	56	86.7	93.3
0.5	59	60	98.3	100.0
1.0	60	59	100.0	98.3

Table 5: Effects of $Na_2S_2O_3$ added to the medium on browning inhibition in loquat leaf culture

	No. of browning		Browning rates (%)	
Concentration ($mg\ L^{-1}$)	Light	Dark	Light	Dark
0.05	31	38	51.7	63.3
0.1	29	40	48.3	66.7
0.3	21	21	35.0	35.0
0.5	36	37	60.0	61.7
1.0	43	50	71.7	83.3

Table 6: Browning effects of CK

	No. of browning		Browning rates (%)	
Codes	Light	Dark	Light	Dark
1	35	37	58.3	61.7
2	32	35	53.3	58.3
3	36	38	60.0	63.3

Each treatment was performed with 60 explants in 15 conical flasks. Explant browning rates were recorded every 2 days, media were renewed once a week and each experiment was replicated for three times.

RESULTS

Effects of explant immersion with bleaching powder, Vc and citric acid: Explants began to show browning during the immersion process with bleaching powder, Vc and citric acid. Browning became serious 3 days later, the color of medium contacting explants became browning on the 12th day. Explant growth was not observed. Table 1 showed that all the three agents were not able to inhibit the browning of loquat leaf explants. From 20 min on, the browning rates increased with the extension of immersion time; the browning rate reached 80% at 90 min. No significant difference was found between light and dark culture.

Effects of PVP added to the medium on browning inhibition: Browning of explants began on the day when they were plated on the medium. Most of the explants turned brown on the 3rd day. About 9 days later, some brown substances accumulated at the edges of explants, some explants even died. Table 2 showed that PVP was not effective on browning control in both light and dark culture.

Effects of $AgNO_3$ added to the medium on browning control in loquat leaf culture: A few explants turned brown on the day when they were plated. About 7 days later, explant browning increased but no brown substances appeared. Table 3 showed that the effects of $AgNO_3$ on browning control was obvious and 0.3 mg L^{-1} $AgNO_3$ was the best at which a lowest browning rate (23.3%) was achieved.

Effects of AC added to the medium on browning inhibition in loquat leaf culture: Table 4 showed that browning began on the 1st day, most explants turned brown 5 days later and became serious. AC had no effect on browning inhibition in both light and dark culture.

Effects of $Na_2S_2O_3$ added to the medium on browning inhibition in loquat leaf culture: Browning started 3 days later, then rapidly sped up, brown substances appeared at the edges of the explants on the 14th day. From Table 5, it can be seen that 0.3 mg L^{-1} $Na_2S_2O_3$ was the most effective on browning inhibition in both light and dark culture. Light culture seemed to be in favour of browning control to some extent.

Browning effect on medium adding no antioxidative agents: Browning appeared the next day after platting to the medium, most of the explants turned brown. Light reaction had better effect than dark. Therefore, light training may be more effective to restrain the browning phenomenon of loquat leaves.

DISCUSSION

Basically, there are two main direct reasons for browning in the process of plant tissue culture: the first one is programmed cell death caused by environmental stress or natural necrosis; the other one is the formation of quinones from phenolic compounds in plant cell under the effect of polyphenol oxidase (Gao, 1999). In callus induction of loquat leaf culture, browning seriously affects the callus production. The basic reason is that there are many phenolic substances exsiting in the tissues of explants, oxidation reaction will happen under appropriate pH, temperature and polyphenol oxidase and then poisonous substances such as lignin, tannins and pigment will be produced (Zhou *et al*., 2000; Zhang *et al*., 2004) and the incisions of explants quickly turn brown at last. If these quinones compounds spread into medium, they will restrain the activity of other enzymes and poison the explants, medium will be also polluted (Rathore *et al*., 1991).

In this study, factors except medium were taken into consideration, the unified basical medium was; MS+6-BA 1.0 mg L^{-1} + 2, 4-D 0.5 mg L^{-1} + Sucrose 30 g L^{-1} + Agar 7.5 g L^{-1}. Media were renewed every 7 days in order to scatter the accumulation of phenolic compounds around the explants in time. At the same time, changes of pH can also change the combining sites of phenolic and polyphenol oxidase which can influence the activity of enzymes (Jianjun *et al*., 2002). Generally speaking, acidic environment goes against the occurrence of browning. Low temperature suppresses the activity of phenol enzymes. In the early period, explants cultured under a lower temperature (15-20°C) can also obviously reduce the browning rates (Liu *et al*., 2008). In this research, the pH was controlled 5.8-6.0, culture temperature 18±2°C, light intensity 2000 Lx. Treatments were carried on under the same conditions to ensure the optimization of anti-browning agents and adsorbents.

Zhang and Ling (1995) thought that Ag^{+} as a kind of ethylene activity inhibitor can not inhibit the synthesis of ethylene but disturb ethylene interfering with the synthesis of biogenic polyamines. PVP can combine with the OH in phenolic compounds and has specific adsorption to phenolic compounds (Loomis, 1974), it can also combine with the enzyme substrate and has certain inhibition to the activity of polyphenol oxidase (Loomis and Battaile,1966). But in this study, PVP showed no obvious effect on browning inhibition of cultured loquat leaves. The researchers thought that it may have something to do with the specific adsorption of PVP. AC can significantly reduce the browning of explants in pear and banana (Jianjun *et al*., 2002; Huang *et al*., 1999). But AC showed no positive effect but aggravated the browning in this study. The reason might be due to the non-substitutable adsorption of AC: it adsorbs the phenolic substances and also adsorbs the nutrients and plant growth regulators, thus it prompts accelerated aging and death of explants.

Bleaching powder, Vc and citric acid have certain inhibitive effect on PPO which is the most important enzyme in enzymatic browning and its activity is a key factor for explant browning (Tale *et al*., 1964). But in this study, with the increase of immersion time, the browning situation aggravated. It might be because during the process of regularly shaking, the explants were slightly damaged or the cut edges of explants were oxygenated because of the exposure in the air.

Taken together, plant tissue browning phenomenon is caused by various factors such as genetic background, physiological status, nutrition, medium composition, salinity, plant growth regulators, culture temperature, light, pH and so on (Ma *et al*., 2006). Developing a set of effective anti-browning measures in loquat tissue culture is an intractable task in the future.

CONCLUSION

Browning was not alleviated by explant immersion in bleaching powder, citric acid and Vc. The addition of AC to medium was not effective in browning control. On the contrary, browning became more serious on medium added AC. Dark culture did not significantly reduce browning. Nevertheless, medium added 0.3-0.5 mg L^{-1} $AgNO_3$ showed a comparatively satisfactory effect on browning inhibition.

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