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RESEARCH ARTICLE

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Studies on browning problem and phenols content on shoots of Yali, Aikansui and Abbe Fetel pears for *in vitro* culture

Abstract Yali is one of the best pear cultivars cultivated extensively in China and other countries. However, mortality rates of explants during the initial phase of tissue culture were found to be very high during the summer particularly from the shoot tip explants. A thorough investigation on the browning problem of Yali Aikansui and Abbe Fetel pears was done and their control measures are suggested in this paper. Emphasis has been given to the Yali pear. Shoot explants were collected from the field as well as from the plantlets grown *in vitro* during different months and observed for browning. The explants were excised and cultured in Murashige and Skoog (MS) solid and liquid medium as needed. The cultures were maintained in the dark as well as in a 16/8 hours light/dark photoperiod regime as needed and were kept in a growth room at $(25 \pm 2)^{\circ}$C temperature. According to the experimental results, Yali was found more severely infected by browning than the other two cultivars. Similarly, it was also found that, the intensity of browning was less during spring and increased with time and reached the maximum during summer. Shoot tips of Yali were found more severely infected by browning than the second node and other nodes. This accelerated the mortality rate up to 81% of shoot explants during the summer months of July and August. Browning was greatly influenced by water-soluble polyphenols, more of which were found in the shoot tip of explants collected from the field (significantly higher at $P \leqslant 0.05$ and $P \leqslant 0.01$). Total polyphenol contents were found less in the explants from plantlets grown in vitro, and less browning of medium appeared resulting in a lower mortality rate of explants. Collections of explants during early spring ($P \leqslant 0.05$ and $P \leqslant 0.01$) and use of other nodes rather shoot tip because explants ($P \leqslant 0.05$ and $P \leqslant 0.01$) were found to be better in preventing the browning problem. Finally, for curative measures to control browning, use of ascorbic acid at the rate of 100 mg·L^{-1} ($P \leqslant 0.05$ and $P \leqslant 0.01$), 0.02% polyvinyl pyrrolidone (PVP) in the culture medium ($P \leqslant 0.05$), 96 hours dark treatment of other nodes ($P \leqslant 0.05$), and 12 hours cold treatments of explants at 4°C ($P \leqslant 0.05$ and $P \leqslant 0.01$) prior to sterilization of explants, were found to be the best methods to control browning and therefore to increase the survival rate of cultured explants of the Yali pear.

Keywords Abbe Fetel, Aikansui, Yali, browning problem, explants, *in vitro* culture, pear, phenols

1 Introduction

Propagation of plants by the tissue culture method is very common in many plant species including fruit crops like pear. However, sometimes during *in vitro* culture of some plant species, the media will become brown and the explants unable to grow further and eventually die. Some explants leach some phenolic substances or secondary metabolites from cut surfaces, which oxidize later and turn the media brown and is toxic to the explants (Aliyu, 2005). Browning of media is common especially from tree species and mature tissues from the woody species. According to Mager and Harel (1979), in the normal tissue, no browning happens, because Polyphenol Oxidase (PPO) and phenolic compounds are separated by a membrane structure and if the structure were to be broken browning happens. Likewise, according to Ju et al. (1988), in the normal cells, polyphenolic compounds are in vacuoles, while the PPO is located in the cytoplasm and if compartmentation were to be broken, browning will happen. Although browning of medium is a serious problem of many plant species for tissue culture, it is an important phenomenon in plants. According to Chen et al. (1997), polyphenol has some regulatory effects on

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the growth and development of plants, disease resistance, and induction of gene expression, signal transduction, biological nitrogen fixation and UV ray absorption.

The problem of browning during tissue culture was reported in many plant species including fruit trees like cashew (Aliyu, 2005), litchi (Chandra and Padaria, 1999), guava (Meghwal et al., 2000), banana (www.comfsm.fm/library/ digitallibrary/kutty.html), avocado (Castro et al., 1995), date palm (www.fao.org/DOCREP/006/Y4360E/y4360e09.htm), and pear (Gao et al., 2003; Ju, 1987; Ji et al., 1998; Li and Qiao, 2001; Li et al., 1994; Yan and Li, 1998). Within the plant species, different varieties differ greatly with each other in terms of the browning problem (www.rirdc.gov.au/reports/WNP/00-36.pdf). This phenomenon is very common in both Asian as well as in the European pears.

Success or failure of tissue culture largely depends on browning of the culture medium especially in fruit trees like pears. In this study, we have focused on the browning problem in the Chinese pear Yali (*Pyrus bretschneideri*), together with two other different species, the Japanese pear Aikansui (*Pyrus pyrifolia*) and the European pear Abbe Fetel (*Pyrus communis*). This study aims to investigate the browning problem of pears particularly Yali and to eventually find and suggest the best ways to overcome the problem.

2 Materials and methods

2.1 Plant materials

Newly grown shoots of about 8-cm long of Yali, Aikansui and Abbe Fetel pears were taken from the fruit orchard located on the Northern side of the West Campus of the Agricultural University of Hebei, Baoding, China. The mother plants were about 5 years old. The samples were taken three times during July 2006 and April and May 2007, thus the trials were conducted thrice to compare the seasonal differences in browning in Yali. Phenol content was examined only once during April 2007. Similarly, newly grown shoots of Yali, Aikansui and Abbe Fetel were taken from the fruit orchard as well as from *in vitro* grown plantlets during July 2007 in order to analyze total phenol content.

2.2 Excise of explants and sterilization

After the collection of explants from the field, they were kept under running tap water for about an hour and the shoots were cut into three different pieces; as shoot tip, second node and other nodes simultaneously. At least one node was kept in each piece of explant for culturing. All the explants were collected in three different jars and labeled according to the explants used as: shoot tip, second node, and other nodes. Then they were washed with distilled water three times before sterilizing for culturing. All the explants were sterilized initially by using 0.1% mercuric chloride ($HgCl_2$) for 7 minutes and then with 75% ethyl alcohol for 30 seconds. To keep the explants free from the hazardous effects of chemicals they were all subsequently rinsed three times in sterile distilled water. After final sterilization, they were kept in sterilized petri dishes and the tip and bottom of the explants were trimmed to make about 1.5-cm long explants and finally cultured for growth. To measure the total phenol contents, shoots from the field were cleaned under running tap water for only about 15 minutes before grinding.

2.3 Growing medium

Murashige and Skoog (MS) full strength liquid and solid growth media supplemented with organic compounds like, thiamine hydrochloride, pyridoxine hydrochloride, amino acetic acid, glycine and inositol at the rate of 0.1 $mg \cdot L^{-1}$, 0.5 $mg \cdot L^{-1}$, 0.5 $mg \cdot L^{-1}$, 2.0 $mg \cdot L^{-1}$ and 100.0 $mg \cdot L^{-1}$ were used in this experiment. Solid media was solidified with 6 $g \cdot L^{-1}$ agar and 30 $g \cdot L^{-1}$ sucrose was added as a carbon source. Instead of agar in the liquid medium, small pieces of sponge were used to keep the explants upright in the conical flask. Twenty-five mL of medium in each conical flask were used in all experiments.

2.4 Use of phytohormones and sterilization

6-Benzylaminopurine (BA) at 1.0 $mg \cdot L^{-1}$ together with 0.1 $mg \cdot L^{-1}$ indole-3-butyric acid (IBA) were used for shoot proliferation. After mixing the phytohormones in the medium, the pH of the medium was adjusted to 5.8 by using litmus paper of pH range 5 to 9 before sterilization. pH was adjusted by using NaOH drop by drop. All the media were autoclaved at 120°C (0.12 kPa pressure) for 20 minutes for sterilization. All other glassware, utensils and distilled water were heat sterilized in an autoclave at 120°C (0.12 kPa pressure) for 45 minutes.

2.5 Use of ascorbic acid and polyvinyl-pyrrolidone (PVP) and cold treatment

100 $mg \cdot L^{-1}$ filter sterilized ascorbic acid was added to the MS solid medium after heat sterilization to conduct the experiment on ascorbic acid to control browning. Likewise, 1 $g \cdot L^{-1}$ and 200 $mg \cdot L^{-1}$ PVP were added to the MS medium to make a concentration of 0.1% and 0.02%, respectively. Similarly, explants were kept for 12 hours at 4°C prior to sterilization in a common refrigerator to conduct the experiment on cold treatment. These experiments were conducted during the month of

May in 2007 and explants were selected from the middle portion of the shoot from the field.

2.6 Experimental design

Each treatment was replicated five times. One 50-mL conical flask with four explants was considered as a replication. Altogether, 100 explants in 25 conical flasks were used in each treatment. Likewise, for liquid culturing, dark culturing, ascorbic acid treatment, cold treatment and PVP treatments, 25 explants, five in each replication with five replications, were made and cultured for different durations of time. Likewise, for the analysis of phenol content, shoots collected from the field were divided into 2 parts, i.e. shoot tip and shoot base, and each was replicated 3 times. Shoots collected from *in vitro* plantlets were not divided; instead, whole plantlets were used and replicated thrice. About one gram of shoot sample (fresh weight) without leaves and shoot apex were used as a sample to measure the water soluble and water insoluble phenols.

2.7 Tissue culture conditions

All cultures were maintained at a temperature of (25 ± 2) °C under a 16/8 hours light/dark photoperiod regime. The light was provided by cool white fluorescent tubes at the cultured flask surface. During the dark treatment, the cultured explants were kept in continuous darkness by covering cultured flasks with thick black cloths and kept inside specially designed racks. Likewise, liquid culture was conducted with the MS liquid medium for 48 hours and later transferred into solid media and cultured for another 10 days. Similarly, during the dark treatment, explants were kept in MS solid media in continuous darkness for 48 hours, 96 hours and finally 144 hours and then they were kept in normal light regimes, i.e. a 16/8 hours light/dark photoperiod. In a similar manner, for cold treatment, all 25 explants were kept for 12 hours inside an ordinary refrigerator at a temperature of 4°C before sterilization.

2.8 Browning problem

One hundred newly cultured explants from 25 conical flasks were randomly selected by visual observation to record the browning problem for check treatment (CK).

2.9 Record keeping

The dead and surviving explants and browning of the media were recorded at 2-day intervals for 10 days. The cultured medium in which the explants died due to heavy browning was considered as a browning problem. The numbers of dead and surviving explants were determined by visual observation.

2.10 Extraction and measurement of phenols

Two types of polyphenols, water-soluble phenols and water insoluble phenols were extracted and measured and analyzed differently with the help of a spectrophotometer (Spectra) with optical density value at 700 nm wavelengths (OD_{700}) and with distilled water as CK. Methods explained by Ju (1989) were used with some modifications for the extraction of polyphenols from the *in vitro* grown shoots and field materials of the selected three varieties of pear. p-hydroxybenzoic acid was used to plot the standard curve as described by Ju (1989).

2.11 Chemicals used

Several types of chemicals were used at different concentrations for the extraction of phenols from the shoots, which were as follows;

1) Sodium carbonate anhydrous A.R. (Na_2CO_3) = 1 g·50 mL^{-1}
2) Sodium Hydroxide (NaOH) = 2 mol·L^{-1}

} 1 + 2 = Solution ①

3) Cupper sulfate ($CuSO_4$) = 0.5 g·100 mL^{-1}
4) Sodium L-tartrate dihydrate = 1% (1 g·100 mL^{-1} distilled water)

} 3 + 4 = Solution ②

5) 50 mL solution ① + 1 mL solution ② = A solution
6) Folin-Ciocalteu's phenol reagent : distilled water (1 : 3) = B solution
7) Spirit = 400 mL
8) Trichloroacetic acid (TAA) = 10% (100 mL distilled water + 10 g trichloroacetic acid)
9) Diethyl ether
10) Methanol = 50% (100 mL distilled water + 100 mL methanol)
11) Hydrochloric acid (HCl) = 1 mol·L^{-1}
12) Sodium Hydroxide (NaOH) = 1 mol·L^{-1}

2.12 Methods used to measure water-soluble polyphenols

- Initially, about 1 gram of fresh tissue samples were measured on an electronic balance and 5 mL of spirit and 2.5 mL of TAA and all shoot materials were put into a mortar and pestle and ground at a temperature below 4°C by using ice in a tray. After grinding, the liquid was poured into 10 mL plastic vials.
- All the ground samples were centrifuged at 10000 r·min^{-1} for 3 minutes at 4°C temperature in a centrifuge machine.

• Upper liquid without any tissues and fibers were transferred into another 10-mL vial.
• Only 1.5 mL of liquid was placed into another 10 mL vial and 3.5 mL of TAA was added to make a total volume of 5 mL and kept at room temperature in darkness for 24 hours.
• After 24 hours, all the samples were again centrifuged at 10000 r·min^{-1} at room temperature for 15 minutes.
• After the centrifugation, a 1/15th mL sample was diluted with 1/14th mL of distilled water, 3 mL of A solution and 1 mL of B solution.
• After waiting half an hour at room temperature, OD_{700} values were measured at 700 nm wavelength one by one with distilled water as CK.

2.13 Methods used to measure water insoluble polyphenol

• After grinding and centrifuging the samples the first time at 10000 r·min^{-1}, all the liquid was discarded and only the remaining undissolved tissues and fibers were transferred into another 10 mL plastic vial and cleaned with 2 mL of Diethyl Ether followed by 2 mL of distilled water and finally with 2 mL of methanol one by one.
• After thoroughly cleaning the sample, 3 mL of NaOH (2 mol·L^{-1}) was poured and thoroughly mixed and kept at 70°C for 20 hours in a hot water bath.
• After 20 hours, the samples were centrifuged at 10000 r·min^{-1} for 15 minutes at room temperature.
• All the liquid was transferred into another 10 mL vial and the remaining tissues and fibers were thoroughly washed 3 times with 0.5 mL of NaOH (1 mol·L^{-1}) each time and thoroughly mixed.
• HCl (1 mol·L^{-1}) was used to adjust pH to 8.
• After adjusting the pH, 1/15th mL of sample was diluted with 1/14th mL of distilled water, 3 mL of A solution and 1 mL of B solution.
• After waiting half an hour at room temperature, OD_{700} values were measured at 700 nm wavelengths with distilled water as CK.

2.14 Statistical analysis

The statistical package DPS (Chinese version) was used to analyze the data by using the Duncan test at $P \leqslant 0.05$ and or $P \leqslant 0.01$ level of significance. The bars in figures and in tables with the same letters are not significantly different while different letters indicate that the variation is significant. Likewise, small letters indicate the significance level at 5% ($P \leqslant 0.05$) and capital letters indicate significance level at 1% ($P \leqslant 0.01$).

2.15 Definitions

Shoot tip explants were considered as explants of about 3-cm long from the tip of the newly grown shoots with a few nodes and internodes. Likewise, nodes of about 2-cm long below the shoot tip were considered as the second node all of which only contain one node. Similarly, the other nodes were considered to be the part below the second node and about 2-cm long all of which also only contained one node. Explants were also divided into two parts; shoot tip, about 3-cm long at the tip of the newly grown shoots; and shoot base, below the shoot tip at about 2-cm long, to conduct experiment to measure polyphenols from the field materials.

3 Results and discussion

3.1 Observation on browning

During shoot proliferation of the three varieties of pears, in an observation during July in 2006, it was found that, Yali was the most susceptible variety to browning followed by Aikansui, and Abbe Fetel. 81%, 45% and 9% explants were infected severely by browning in Yali, Aikansui and Abbe Fetel, respectively (Fig. 1).

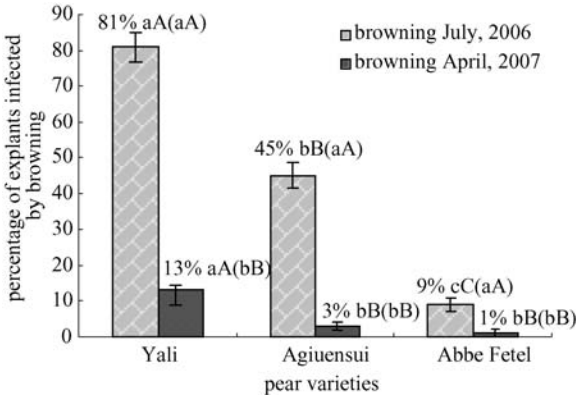

Fig. 1 Different varieties of pear affected by browning with their percentage in different seasons
Note: Letters out of bracket were compared among the different varieties and within brackets are compared with different months within the same variety; Bars with same letters are not significantly different, small letters have significant differences at $P \leqslant 0.05$ level, capital letters have significant differences at $P \leqslant 0.01$ level.

All three varieties were significantly different from each other at both levels of significance $P \leqslant 0.05$ and $P \leqslant 0.01$ (Fig. 1). It was also observed that, the number of surviving shoots depended on the browning intensity. In this observation, it was found that, all explants of all three varieties with browning problem died and the remaining 19%, 55% and 91% of explants of Yali, Aikansui and Abbe Fetel without the browning problem survived during July in 2006.

The result of the same experiment during April in 2007 was drastically different from the result of July in 2006. During this period, only 13%, 3% and only 1% of explants

were infected by browning in Yali, Aikansui and Abbe Fetel pears, respectively. Similarly, in another observation during the first week of May in 2007, 60% of Yali explants were found to be infected by browning. The result showed that, of the Asian pear varieties, Yali is the most susceptible to browning followed by Aikansui and finally the European pear variety Abbe Fetel, which was considered more resistant to the browning problem. Likewise, it was also found that the browning problem was less during early spring and the intensity increased with time and reached the highest during summer as temperature rose in Baoding (Fig. 2). Smith, (www.rirdc.gov.au/reports/WNP/00-36.pdf) explains that, the browning problem may be influenced by the age of the parent material and the time of collection of explants. Gao et al. (2003) found that May was the best month for explant inoculation as during this time browning of the media was less as in the case of pear variety Xinli No. 7, which was similar to our result. Contrary to our result, in the case of the Cangxili pear (*Pyrus pyrifolia*), Li and Qiao (2001) found variations in the survival rate with the season of the explants collection, as the highest browning rate occurred in explants collected in April, and from May onwards, the browning rate decreased, and it was reduced to 0 in October. However, it was the opposite in our case.

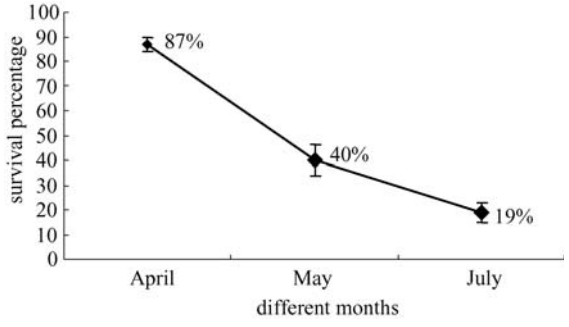

Fig. 2 Survival rate of explants of Yali in different months

3.2 Effect of liquid culture on browning

An experiment was conducted in July 2006 on the Yali pear with an MS liquid medium to find out the intensity of browning during the course of time and the effects of the liquid medium on the rate of survival of explants and explant types. According to the result (Fig. 3), after 48 hours in an MS liquid medium and 10 days in an MS solid medium, 20% of explants survived from which only 5% of explants were from the second node and, 15% were from the other nodes and no single shoot tip survived. The survival rate of explants from the other nodes was significantly higher ($P \leqslant 0.05$ and $P \leqslant 0.01$) than that from shoot tip and second node. The survival rate of explants of shoot tip and second node was statistically not significant even at $P \leqslant 0.05$. It was also found that, in a visual observation, intensity of browning was more in the shoot

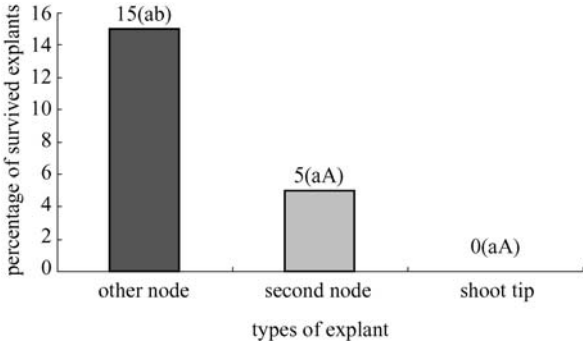

Fig. 3 Survived explants (%) from different types of explant cultured in liquid MS medium
Note: Bars with same letters are not significantly different; small letters have significant differences at $P \leqslant 0.05$ level; capital letters have significant differences at $P \leqslant 0.01$ level.

tip, medium in second node and less in other node. This experiment clarifies that, the browning problem was more serious in the shoot tip followed by the second node and the other node, hence the survival rate of explants was higher from the other node followed by the second node.

An experiment was conducted to know the intensity of browning in liquid medium during different intervals of time. From this experiment it is found that, browning could be seen by the naked eyes even after 6 hours of culturing and the intensity increased with each hour. According to Fig. 4, the color of the medium after 48 hours (right corner) was the darkest followed by the medium after 24 hours of culturing (second last). Likewise, the color of the medium after 12 hours of liquid culturing (middle) was lighter than that of 24 hours, and 6 hours of culturing (second from left) was lighter than that of 12 hours. CK was the only medium kept for 48 hours without any explants, which was white in color (left corner). It reveals that browning starts immediately after culturing in the media, and increases in intensity with time, and reaches the maximum level a few days later.

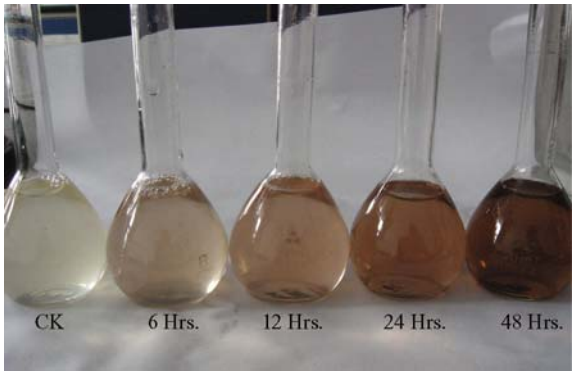

Fig. 4 Browning of medium in CK, 6, 12, 24 and 48 hours after culturing in liquid media

According to Ye et al. (2004), normally in the liquid medium, poisonous substances may disperse quickly so

explants may not be hurt so seriously than that in a solid medium. However, in our case the total survival rate of explants in liquid medium was only 20%, which was very similar to the survival rate of the ordinary solid media of 19%, which were not significantly different from each other.

3.3 Effect of dark treatment on browning

Another experiment was carried out to determine the effect of dark treatment on browning and hence the survival rate of different types of explants of Yali in an MS solid medium. As for the duration of dark treatment, according to the result, all shoot tips in all treatments died and there were no significant differences between any treatments with CK (no dark) on any level due to heavy browning during culturing (Fig. 5). The result was the same in liquid medium. Likewise, 4%, 12%, 8% and 8% of explants survived from the second node in CK, T-1, T-2 and T-3 treatments respectively. Second nodes were also not significantly different from shoot tip, CK and other treatments. Finally, 20%, 24%, 28% and 24% of explants survived from the other node with CK, T-1, T-2 and T-3 respectively as in Fig. 5. The other node with T-2 was the only treatment that was significantly different from CK and T-1 and T-3, at the $P \leqslant 0.05$ level of significance. Therefore, only T-2, i.e. 96 hours of dark treatment of the other node, was the best treatment to control browning of explants.

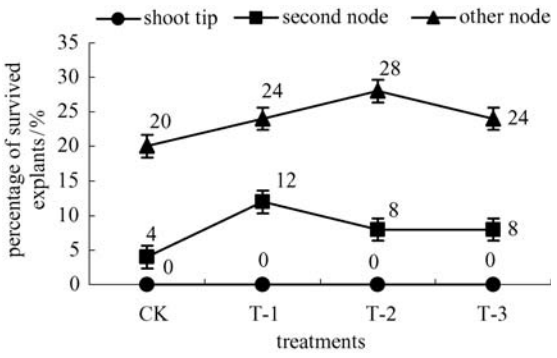

Fig. 5 Survival rate of explants during dark treatment
Note: CK = no dark, T-1 = 48 hours dark, T-2 = 96 hours dark, T-3 = 144 hours dark.

As for the explant types, the other node with CK was significantly different from the shoot tip and second node at both $P \leqslant 0.05$ and $P \leqslant 0.01$ levels of significance. This shows that the survival percentages of explants within the explant types were higher from the other node as in all other experiments. It also shows that, the browning problem was severe in the shoot tip, which was not possible to control by dark treatment alone.

Some scientists have worked with dark treatment for different plant species and were able to reduce the browning problem significantly. Zhao et al. (2006) found that dark pretreatment for 10 days for explants of *Phalaenopsis* could decrease the browning rate significantly. Likewise, Zhang et al. (2003) were able to control the browning rate of cultured shoot explants of the walnut with 2 $g \cdot L^{-1}$ activated charcoal (AC) after dark culturing for 7 days. However, in our case with Yali, only 4 days of dark treatment was enough to increase the survival rate of explants significantly. According to Biedermann (1987), dark culturing is good for alleviating browning in *Magnolia* hybrids. Marks and Simpson (1990) were able to reduce phenolic oxidation while culturing initiation *in vitro* after the exposure of field grown stock plants to darkness or low level of irradiance.

3.4 Use of ascorbic acid to control browning

Use of ascorbic acid in the growing medium was very effective to control browning especially in the month of May. According to the result (Table 2), only 8% explants of Yali were infected by browning and 92% of explants survived by using 100 $mg \cdot L^{-1}$ ascorbic acid in the MS growing medium, which had a very high survival percentage compared to the CK from which only 40% of explants survived during the same period. The result was significantly higher in both levels of significance at $P \leqslant 0.05$ and $P \leqslant 0.01$ compared to CK. Several researchers have explained the effective control of browning by using ascorbic acid. He et al. (1995) treated banana explants with ascorbic acid before culturing and lessened the percentage of browning. Use of 280 $mmol \cdot L^{-1}$ of ascorbic acid also reduced browning of the medium in cashew (Aliya, 2005). Similarly, in an experiment conducted by Peng et al. (2007) on *Bromelia* spp., they found more

Table 1 Mortality of explants due to browning in Yali cultured in liquid MS medium and in dark

type of explant	mean number and browning rate/%				
	liquid	CK	T-1	T-2	T-3
shoot tip	20.0 (100) aA [aA]	20.0 (100) aA [aA]	20.0 (100) aA [aA]	20.0 (100) aA [aA]	20.0 (100) aA [aA]
second node	19.0 (95) aA [aA]	19.2 (96) aA [aA]	17.6 (88) bB [aA]	18.4 (92) bB [aA]	18.4 (92) bA [aA]
other node	17.0 (85) bB[aA]	16.0 (80) bB [abA]	15.2 (76) cC [abA]	14.4 (72) cC [bA]	15.2 (76) cB [abA]

Note: CK = no dark, T-1 = 48 hours dark, T-2 = 96 hours dark, T-3 = 144 hours dark; Letters out of bracket compared vertically with type of explants used and within [bracket] are compared horizontally for significant i.e. compared with time; Same letters are not significantly different, small letters have significant differences at $P \leqslant 0.05$ level, capital letters have significant differences at $P \leqslant 0.01$ level; There were no effects of all three dark treatments on the control of browning, no significant differences between no dark and dark treatments.

Table 2 Polyphenols content of *in vitro* grown saplings

variety	mean fresh weight/(mg·g^{-1})		total phenol content fresh weight/(mg·g^{-1})
	water soluble phenol	water insoluble phenol	
Yali	13.44 aA	14.00 aA	27.44 aA
Aikansui	13.27 bAB	13.82 bAB	27.09 bAB
Abbe Fetal	13.67 bB	14.29 bB	27.96 bB

Note: Same letters are not significantly different, small letters have significant differences at $P \leqslant 0.05$ level, capital letters have significant differences at $P \leqslant 0.01$ level compared vertically with different varieties.

severe problems of browning in *Aechmea fasciata* than in *Guzmania* and *Vriesea charlotte* during *in vitro* culturing. They also concluded that thalamus and inflorescence were unsuitable explants for *in vitro* culturing and browning was controlled by 7 days of dark culturing and medium supplemented with vitamin C at the rate of 200 mg·L^{-1}.

3.5 Role of polyvinyl pyrrolidone (PVP) on browning

Use of PVP in the growing medium was also one of the easiest ways to control the browning. According to the results (Table 2), only 60% of explants survived from 0.1% of PVP not significantly different from CK, however 72% of explants survived by using 0.02% PVP in the growing medium which had a high survival percentage (significant at $P \leqslant 0.05$) compared to CK. According to Wei et al. (2007), the better depressor for lamina (*Pistacia vera*) to restrain browning was PVP, and its best concentration was at 1.3 to 1.5 g·L^{-1}. According to Zhou (2007), soaking the explants of *Aloe saponaria* in PVP (2 g·L^{-1}) solution for 15 minutes showed distinct effects on controlling browning.

3.6 Effect of cold treatment on browning

Cold treatment of explants prior to sterilization in an ordinary refrigerator for 12 hours at 4°C was the easiest and one of the best ways to control browning in the case of Yali pear. According to the experiment result (Fig. 6), only 8% of explants were infected by browning (significantly higher in both levels of significance at $P \leqslant 0.05$ and $P \leqslant 0.01$ compared to CK) and the remaining 92% of explants survived in this treatment during the month of May. Li and Qiao (2001) improved the survival rate of explants by using low temperature treatment before the culturing of explants and with the use of antioxidant 8-hydroxyquinolin (8-HQS) at the rate of 0.002 mL·L^{-1} in the culture medium for the Cangxili pear (*Pyrus pyrifolia*). Zhang et al. (2003) suggested to control browning during the shoot tip culturing of a pellicular walnut by using dark culturing with activated carbon 2 g·L^{-1} at 5°C for 7 days. Liu and Han (1986) did an experiment on walnuts and found that cold pretreatment could reduce browning. According to Zhang et al. (2003), the effective method to anti-browning for shoot tips of walnuts was pretreatment under cold storage at 1–2°C for 24 hours; then dipping in 20% sodium thiosulfate for 20–30 minutes and subsequently washing with tap water for 8–10 hours, and then, culturing in the medium added with PVP 2.0 g·L^{-1}.

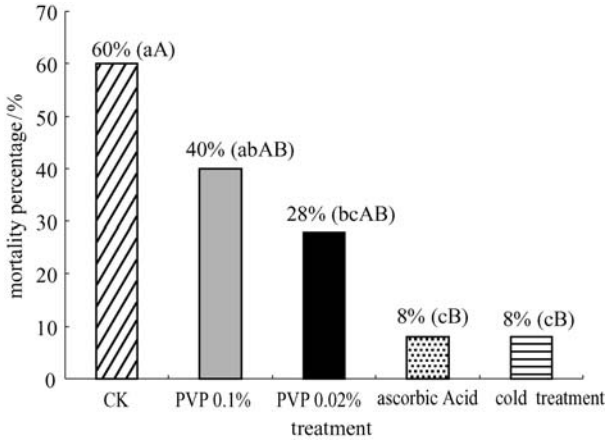

Fig. 6 Mortality of explants due to browning in Yali

3.7 Use of activated charcoal (AC) to control browning

Before sterilization of explants of the Yali pear they were dipped into 1 g·L^{-1} AC for 12 hours and cultured in a solid MS medium. However, all explants showed severe browning and eventually all explants died in April. Likewise, in July 2006, the MS medium was enriched with 1 g·L^{-1} AC and explants were cultured for growth, but all explants again showed high browning and eventually died within a week. Many scientists have suggested the use of AC to control browning, but in our case, it did not work for Yali. According to Aliya (2005), in addition to frequent transfers of cashew explants and the addition of activated charcoal, cultivation in darkness for one week increased the survival rate of explants to 90%. Smith (www.rirdc.gov.au/reports/WNP/00–36.pdf) also suggested the use of AC in the culture medium to control browning effectively for *Ensete*, oil palm and apple and pear cultivars. In order to prevent browning of callusing stem explants of bamboo (*Dendrocalamus latiflorus*), several antioxidants and adsorbing agents were tested by Ramanuja et al. (www.inbar.int/publication/txt/INBAR_BR_03.htm). According to them, the most effective way to control browning was the application of

activated charcoal (2 g·L^{-1}) and incubation in the dark. The former, however, inhibited callus development and higher levels of growth regulators were needed to compensate for the inactivating properties of the activated charcoal.

3.8 Phenol contents in shoots

Another experiment was conducted for the Yali, Aikansui and Abbe Fetel pears to measure the total phenol contents of *in vitro* grown proliferated shoots of about one year old plantlets, and shoots collected directly from the field were compared. According to the result of the experiment (Table 3), water-soluble as well as water insoluble phenol contents of all three varieties were almost similar which were about 13 to 14 mg·g^{-1} of shoots on a fresh weight basis. Total phenol contents were also almost the same, about 27 mg·g^{-1} of shoots on fresh weight basis. Water-soluble and water insoluble phenol content in each of the varieties were not significantly different at any level. However, Abbe Fetel had a significantly high level of water soluble and water insoluble phenol than Yali ($P \leqslant 0.05$ and $P \leqslant 0.01$). Nevertheless, the total quantity of phenols was very low due to which browning did not affect the culture medium and hence no shoots were found dead from all 3 varieties of the *in vitro* grown plantlets.

Table 3 Polyphenols content in the explants collected from the field (mg·g^{-1} fresh weight)

phenol type	variety		
	Yali	Aigunsui	Abbe Fetel
water soluble tip	44.64 aA	13.90 bB	13.15 bB
water soluble base	13.86 aA	13.43 bA	13.34 bA
water insoluble tip	21.17 bAB	22.22 aA	20.90 bB
water insoluble base	21.45 aA	21.24 aA	21.10 aA
total phenols tip	65.81 aA	36.09 bB	34.05 bB
total phenols base	35.31 aA	34.68 abA	34.44 bA

Note: Same letters are not significantly different, small letters have significant differences at $P \leqslant 0.05$ level, capital letters have significant differences at $P \leqslant 0.01$ level compared horizontally with different varieties.

Contrary to this, phenol content of fresh explants, which were collected from the field, was higher compared to shoots from plantlets grown *in vitro*. For shoots collected from the field, they were divided into two parts, shoot tip and shoot base, and phenol contents were measured separately.

According to the result (Fig. 7), water soluble phenol was found high in the shoot tip of Yali, i.e. 44.64 mg·g^{-1} of fresh shoot sample, significantly higher than the shoot tip of the other 2 varieties Aigunsui and Abbe Fetel in both levels of significance, i.e. at $P \leqslant 0.05$ and $P \leqslant 0.01$. However, the other two varieties, Aigunsui and Abbe Fetel were not significantly different from each other. Likewise, water-soluble phenol from the base of Yali was significant only at $P \leqslant 0.05$ compared to the two other varieties. Moreover, water-soluble phenol content at the base was almost the same in all three tested varieties, i.e. about 13 mg·g^{-1} of shoot. Similarly, water insoluble phenol content in all three varieties in the shoot tip and base were a little higher than water-soluble phenol, ranging from 20.90 to 22.22 mg·g^{-1} of fresh shoot. Water insoluble phenol content in the tip of Abbe Fetel was significantly higher at $P \leqslant 0.05$ compared to the tip and base of the other varieties and almost none was significantly different from the others in terms of water insoluble phenol content in the shoot tip and base of all three varieties. In the same manner, total phenol content in all three varieties from shoot tip and shoot base (except Yali's shoot tip) had almost equal amounts of phenol, i.e. from 34.05 to 36.09 mg·g^{-1} of shoot sample. Our experiment confirmed that the shoot tip of Yali from the fresh sample from the field contained high amounts of water soluble phenol, which played a vital role in the browning of explants and finally the survival rate of the explants during the initial period of shoot culturing and proliferation. On the other hand, the samples from *in vitro* grown plantlets of all three varieties had almost the same amount of phenol and the amount was not enough to create browning of

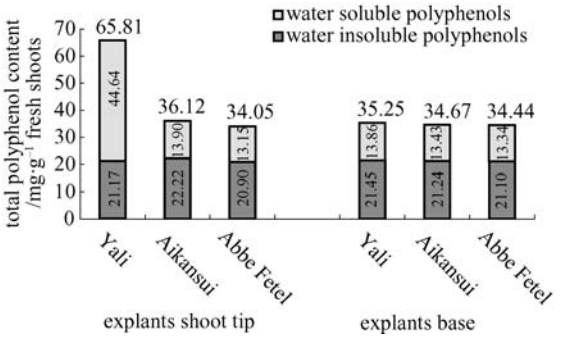

Fig. 7 Total phenol content from the shoot tip and base of explants from the field

the culture medium and hence to kill the explants.

Many scientists have worked to determine the relationship between total phenol content and browning of the culture medium in different plant species and found similar results like ours in the pear. According to Xia (2006), in *Gerbera jamesoniiin*, the phenol contents increased with the increasing degree of browning. The degree of browning of pear tissue had a significant positive correlation with PPO activity and the content of phenolic compounds (Ju, 1987). Similarly, according to Ju (1989), the soluble polyphenol causing browning was mainly chlorogenic acid in pears and others like cinnamic acid, vanillic acid and coumaric acid. Chen (1986) pointed out that, if the genotypes of explants were different, the degree of the browning would be different. In peach fruits, the degree of browning was

closely related to the content of phenolic compounds (Chen and Wang, 1995). Ji et al. (1998) found that, in pear dwarf rootstocks S4, the content of phenolic compounds and the percentage of browning had a significant positive correlation ($r = 0.8815$). Li et al. (1994) found in pear buds and shoot tip culturing that the content of polyphenolic compounds had a positive relationship with the browning percentage, and a negative relationship with the survival percentage.

Similarly, Li and Yan (2001) studied the yearly changes of peroxidase (POD) and PPO and their isozymes in the buds and terminals in the annual shoots of the Jinhua pear (*Pyrus bretschneidari*) and Changxi pear (*P. pyrufolia*). They found that the highest activity of both enzymes appeared in April and the lowest activity occurred in December for the Jinhua pear and in January for the Cangxi pear. POD and PPO activity of both pear species was positively correlated with the browning rate of their terminals and buds as explants in tissue culture in most months of the year. In the same manner, Yan and Li (1998) found that phenol content was interrelated with the browning ratio of the explant of two varieties of *in vitro* grown pears Comgxili (*Pyrus pyrifolia*) and Jin Hua (*P. bretshneideri*). The browning ratio was affected by the phenol content and PPO activity of the explants. Likewise, Li et al. (1994) studied the relationship between the regulation of PPO activities and phenol contents in buds and stems of 5 pear varieties of Yali (*P. bretshneideri*), Pingguoli (*P. sinkia*ngensis), Nanguoli (*P. ussuriensis*), Cangxili (*P. pyrifolia*) and Rimianhong (*P. communis*) during March to September and concluded that the phenol contents in those varieties in September were the highest and those in March were the lowest. This result was similar as our findings.

Different species of pear differ greatly in phenol content in different months of the year. In the Comgxiliv pear (*Pyrus pyrifolia*), the peak phenol content level appeared in May, i.e. 50 $mg \cdot g^{-1}$ (fresh weight), but in Jin Hua (*P. bretshneideri*) its peaks appeared in March and September and was 37 $mg \cdot g^{-1}$ and 38 $mg \cdot g^{-1}$ (fresh weight), respectively (Yan and Li, 1998). Total polyphenol contents during the month of April in 2007 in the case of Yali (*P. bretshneideri*) in our experiment was 65.81 $mg \cdot g^{-1}$ of fresh shoot, which was a bit higher than the Jinhua variety. Li et al. (1994), measured the total phenol content in the buds and stem terminals of the Yali pear in different months and they found that the total phenol content increased with each month from March to September with figures of 27.75 $mg \cdot g^{-1}$ in March, 54.46 $mg \cdot g^{-1}$ in April, 72.61 $mg \cdot g^{-1}$ in May, 33.7 $mg \cdot g^{-1}$ in June, 53.21 $mg \cdot g^{-1}$ in July, 107.99 $mg \cdot g^{-1}$ in August and 179.04 $mg \cdot g^{-1}$ in September (fresh weight), respectively.

Once the shoot has been established *in vitro*, the total polyphenol contents in the tissues will reduce drastically particularly water-soluble polyphenol and hence there is no browning problem, and eventually the mortality rate of explants will reduce steadily.

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