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Micropropagation of 'Wild Pear' *Pyrus pyrifolia* (Burm F.) Nakai.I. Explant Establishment and Shoot Multiplication

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Abstract

The study was undertaken to standardize *in vitro* explant establishment and shoot multiplication technique for wild pear. The highest explant establishment (77.88%) was observed during spring, which was on a par with explant establishment frequency during winter season (76.21%). The lowest explant establishment was observed during rainy season (61.94%). The establishment frequency for shoot tips (69.07%) was significantly less than the establishment frequency for nodal segments (71.95%). The antibrowning agents decreased explant browning, yet they could not eliminate it. The magnitude of the effect of the antibrowning agents depended on the season of explant collection and type of explant. Antibrowning agents were more useful during spring and winter seasons when the incidence of browning was low, in comparison to rainy, summer and autumn seasons when the browning was comparatively high. Though, the highest number of shoots (11.24/ culture) were obtained with 2.0 mg per litre BA, but this BA level did not produce shoots of desirable length (>2.0 cm). BA (1.5 mg per litre) + IBA (0.5 mg per litre) was the best growth regulator combination for shoot multiplication in wild pear as it produced sufficient number of shoots (10.21 shoots per culture) having desirable shoot length. The highest number of shoots per culture (11.20) was obtained with Woody Plant Medium (WPM), followed by MS medium (10.21). The highest shoot length (3.21 cm) was observed with MS Medium and it was on a par with WPM.

Keywords: micropropagation, explant browning, shoot multiplication, wild pear

Introduction

The domestic pears of Asia are derived mostly from *P. pyrifolia* (Burm F.) Nakai, known as Japanese or sand pear. The sand pear, *P. pyrifolia* is the main cultivated species in southern and central China and in Japan. Pears are grown in all the temperate zones of the world. The Himalayan region of India is abundant in the production of temperate fruits, which provides food and nutritional security. In India, the temperate fruits are being grown in India from warm humid sub-tropical plains to cold dry temperate regions. Global climate change is leading to some production problems with temperate fruits. The rise in winter temperatures and poor snowfall in the marginally temperate areas are making them unfit for the production of temperate fruits. On the other hand, there is an increasing opportunity for the exploitation of low chilling varieties of temperate fruits in the marginally temperate and subtropical regions of North-West India. The Punjab state having the subtropical climate known for the production of cereal grains and citrus fruits worldwide is also going towards temperate fruit growing. In India, pear is grown on an area of 23. 000 ha with an annual production of 200.000 mt (Anon., 2003). The chief pear growing areas in India are located in the states of Jammu and Kashmir, Himachal Pradesh, Punjab, Uttaranchal, Arunachal Pradesh, Manipur, Mizoram, Nagaland and Tamil Nadu. In Punjab, area under pear is 2.560 ha, with an annual production of 51.200 mt (Anon., 2006). The selection of some promising low chilling semi-soft pear cultivars from Punjab state namely, Punjab Beauty, Punjab Nectar, Punjab Gold and Punjab Soft, has increased the demand for pear planting material in the entire North-west India.

Pear is usually grafted or budded on pear seedlings or clonal rootstocks. In the subtropical regions of North-West India, wild pear, *Pyrus pyrifolia* (Burm F.) Nakai is the most commonly used rootstock for pear. The trees on this rootstock are semi-vigorous. To the best of our knowledge no other research workers has taken up the work on the micropropagation of 'wild pear', *Pyrus pyrifolia* (Burm F.) Nakai in India or abroad. *In vitro* propagation has been reported for rapid and year-round propagation of pear rootstocks (Dwivedi and Bist, 1997, Shibli et al., 1997 and Yeo and Reed, 1995). The frequency of explant establishment varies with explant type and season of explant collection. Browning of explants cultured *in vitro* is a serious problem that impairs successful micropropagation of woody plants. In "axillary shoot proliferation", cytokinins are utilized to overcome the apical dominance of shoots to enhance the branching of lateral buds from leaf axils. The effective concentration of cytokinins required to reverse apical dominance varies with the culture systems. Therefore, the pres-

ent study was undertaken to standardize *in vitro* explant establishment and shoot multiplication technique for wild pear.

Materials and methods

Shoot-tip and nodal segment explants were collected from current season's growth of wild pear *Pyrus pyrifolia* (Burm F.) Nakai from March to November, 2003. Current season's shoots (12-15 cm) were excised and 1.0 to 1.5 cm long shoot-tip and nodal segment explants were prepared. The explants were rinsed with tween 20 and washed under running tap water for about 5 minutes. The explants were surface sterilized with aqueous solution of mercuric chloride (0.1 % w/v; 4 minutes) and were washed with sterilized distilled water thrice to remove all the traces of disinfectant. These explants were then vertically cultured in culture tubes with 15 ml MS medium (Murashige and Skoog, 1962) supplemented with BA (1.0 mg per litre) and IBA (0.5 mg per litre) plus sucrose 3 per cent, 100 mg per litre inositol and 0.7 % agar. The data on the effect of explant type (shoot tip or nodal segment) and season of explant collection on per cent expant establishment and browning were recorded four weeks after culture. Explants that turned dark brown or black, released compounds from the cut ends that lead to browning of medium and did not sprout after four weeks of culture were recorded as browning. Antibrowning agents viz. ascorbic acid (AA; 500 and 1000 mg per litre); and citric acid (CA; 500 and 1000 mg per litre); Polyvinyl pyrrolidone (PVP; 2500-5000 mg per litre); and activated charcoal (AC; 1000 and 2000 mg per litre) were added to the explant establishment media comprising MS salts supplemented with BA (1.0 mg per litre) and IBA (0.5 mg per litre). The explants (shoot tips and nodal segments; 1.0-1.5 cm long) were cultured on these media during spring (February-March), summer (April-June), rainy (July-August), autumn (September-October) and winter season (November-December). The observation on per cent explant survival was taken four weeks after culturing.

After four weeks, shoots from the established explants were cut and subcultured on MS medium supplemented with varying levels of BA and IBA (Table 3) in 500 ml glass jars containing 50 ml MS medium for shoot multiplication. To determine the effect of different media compositions, the shoots were transferred to MS medium, ½ MS medium, Woody plant medium (Lloyd and McCown, 1980) and modified MS medium (ammonium nitrate reduced to 400 mg per litre). Observations on number of shoots per explant and shoot length were recorded six weeks after fourth subculture.

All the experiments were carried out as completely randomized designs as described by Panse and Sukhatme (1954) and replicated atleast thrice. Ten cultures at each concentration formed one replication. The significance of variation among the treatments was observed by applying 'F' test. The least significant difference (LSD) at p=0.05 was calculated by multiplying standard error with 't' value (p=0.05) at error degree of freedom to compare the means of the treatments. To study the combined effect of two factors, the data were subjected to two-way ANOVA analysis.

Results and discussion

The highest explant establishment (77.88%) was observed during spring which was on a par with explant establishment frequency during winter (76.21%). The lowest explant establishment was observed during rainy season (61.94%). Further, the establishment frequency for shoot tips (69.07%) was significantly less than the establishment frequency for nodal segments (71.95%). There was no significant interaction between explant type and season of explant collection. The data also show that the phenolic browning was the lowest during spring (8.26%) and it was on a par with winter season.

The browning was highest during rainy season (12.27%). Increase in explant browning (Table 1) and endogenous explant contamination (data not presented) from spring to rainy season, which decline considerably till winter season may be responsible for the seasonal variation in explant establishment.

High levels of growth promoting substances and low growth inhibitors in actively growing shoots during spring may be responsible for high explant establishment during spring. Higher phenolic browning in shoot tip as compared to nodal segments in all seasons (Table 1) may be responsible for lesser frequency of shoot tip establishment. *In vivo* synthesis of polyphenolic compounds is reported to vary with growth stages, environmental factors and occurrence of diseases (Vaughan and Duke, 1984). Low browning in spring and winter seasons may be due to low *in vivo* phenolic content as the short day length prevailing during this period has been reported to reduce the *in vivo* phenolics (Chevere et al., 1983). Higher browning in shoot tips than nodal segments may be due to higher phenolics as the shoot tips are actively growing and herbaceous in nature (Singh et al., 2002). The phenolic compounds released from the cut ends of the explants are oxidized by polyphenol oxidase, peroxidases or air. Quinones, the oxidized products are highly reactive and inhibit enzyme activity leading to the death of explants (Hu and Wang, 1983).

The effect of antioxidants (ascorbic and citric acid) and phenol binding agents (polyvinyl pyrrolidone and activated charcoal) on explant browning during different seasons is presented in Figure 1 to 5. Figure 1 shows that in spring season highest explant survival percentage (81.91%) was observed with citric acid (1000 mg per litre) and it was on a par with ascorbic acid (1000 mg per litre), citric acid (500 mg per litre) and polyvinyl pyrrolidone (2500 and 5000 mg per litre). In rainy season, the maximum explant survival (64.59%) was observed with ascorbic acid (1000

mg per litre) incorporation in the explant establishment medium (Figure 3). It was on a par with ascorbic acid (500 mg per litre), citric acid (250-1000 mg per litre), polyvinyl pyrrolidone (2500 and 5000 mg per litre) and activated charcoal (2000 mg per litre). In autumn, maximum explant survival (71.50%) was observed with citric acid (1000 mg per litre; Figure 4). It was on a par with 500 mg per litre) citric acid, ascorbic acid (500 and 1000 mg per litre), polyvinyl pyrrolidone (2500 and 5000 mg per litre) and activated charcoal (2000 mg per litre). There was no significant difference between all the treatments, for explant survival percentage during summer and winter season (Figure 2 and 5).

Addition of antioxidants (ascorbic acid and citric acid) to establishment media has been reported to be effective in preventing oxidation of phenolics (Kumar and Kumar 1998). Wang et al., (1994) have reported the effectiveness of ascorbic acid and citric acid in reducing phenolic browning and enhancing explant establishment in apple. The effectiveness of polyvinyl pyrrolidone (PVP) and activated charcoal in overcoming explant browning in all the pear genotypes may be due to the binding of phenols by polyvinyl pyrrolidone and activated charcoal. Browning of cultures has been effectively controlled by the addition of adsorbants like activated charcoal (Wang et al., 1994) and polyvinyl pyrrolidone (Shibli et al., 1997) in culture media. The relatively poorer effectiveness of antibrowning agents during summer, rainy and autumn seasons in comparison to spring and winter seasons may be due to higher *in vivo* phenolic content in the stock plants.

Shoots from the established cultures were cultured on MS medium supplemented with varying levels of BA, IBA and their combinations (Table 2) for shoot multiplication. Initially, shoot growth was very slow at all the growth regulator combinations tested. However, after two subcultures of forty days each on respective media, two to three axillary shoots arose from the base of cultured shoots. The excised shoots (1-2 cm long) took longer time to start

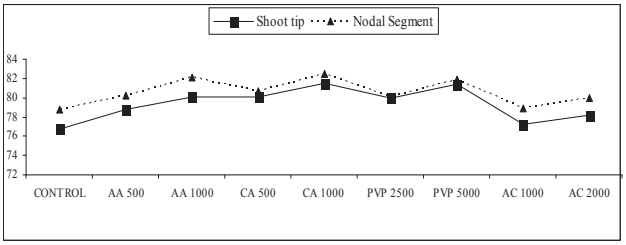

Figure 1 Effect of explant type and antibrowning agents (mg/l) on explant survival during spring season season

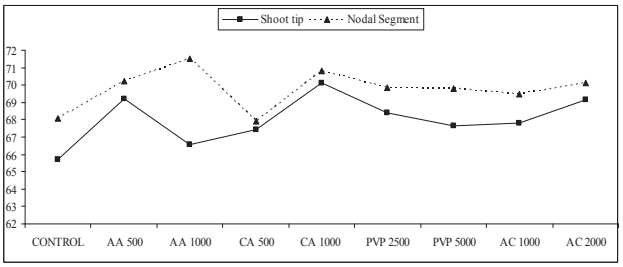

Figure 2 Effect of explant type and antibrowning agents (mg/l) on explant survival during summer season

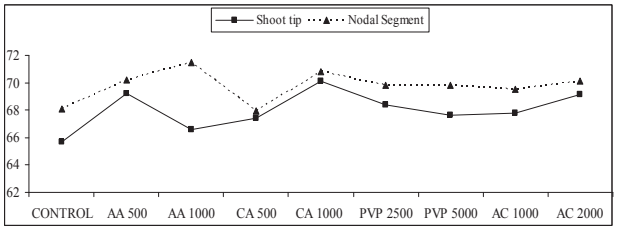

Figure 3 Effect of explant type and antibrowning agents (mg/l) on explant survival during rainy season

Table 1 Effect of explant type and season of explant collection on explant establishment (%) and phenolic browning (%)

	Explant establishment (%)			Phenolic browning (%)		
	Shoot tip	Nodal Segment	Mean	Shoot tip	Nodal Segment	Mean
Spring season	76.73 (61.15)	79.03 (62.74)	77.88 (61.94)	9.43 (17.81)	7.10 (15.38)	8.26 (16.06)
Summer season	65.70 (54.14)	69.44 (56.42)	67.57 (55.28)	11.59 (19.83)	9.40 (17.80)	10.49 (18.81)
Rainy season	60.87 (51.26)	63.02 (52.53)	61.94 (51.89)	12.44 (20.63)	12.10 (20.31)	12.27 (20.31)
Autumn season	66.91 (54.86)	71.03 (57.42)	68.97 (56.14)	12.07 (20.27)	10.27 (8.62)	11.17 (18.62)
Winter season	75.16 (60.10)	77.27 (61.51)	76.21 (60.81)	9.67 (18.02)	9.02 (17.44)	9.34 (17.44)
Mean	69.07 (56.30)	71.95 (58.12)		11.04 (19.31)	9.57 (17.91)	
LSD (0.05)		Explant (A) : (1.08) Season (B) : (1.71) A x B : NS			Explant (A) : (1.36) Season (B) : (2.16) A x B : NS	

Figures in parentheses are the transformed values

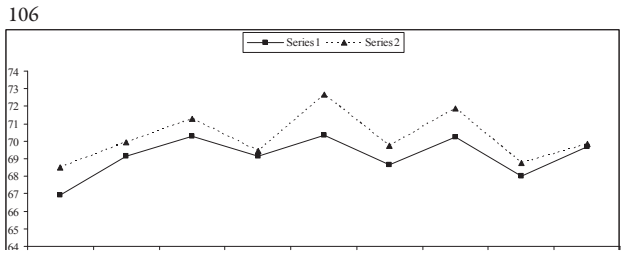

Figure 4 Effect of explant type and antibrowning agents (mg/l) on explant survival during autumn seasons

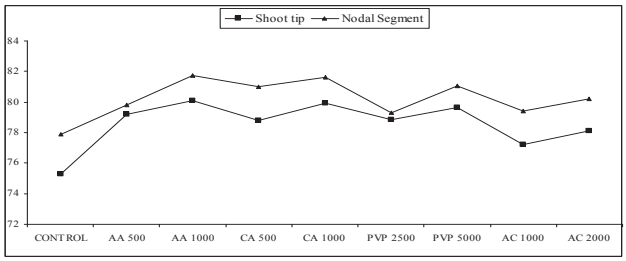

Figure 5 Effect of explant type and antibrowning agents (mg/l) on explant survival during winter season

AA: Ascorbic acid, CA: Citric acid, PVP: Polyvinyl pyrrolidone and AC: Activated charcoal

growth and proliferation than the pieces of proliferated shoot mass on proliferation medium. At each subculture the proliferated shoot mass was cut in to 4 –5 pieces having the parent tissue. Hence, the rate of shoot multiplication refers to the average number of propagules derived from a parent culture after fouth subculture. The initial lag phase during the shoot multiplication stage was due to slow growth of cultures and it is earlier reported in *Pyrus* spp. In *Pyrus pyrifolia*, shoot proliferation was delayed by five months (Bhojwani et al., 1984). Similarly, 7-8 months lag period has been reported in *P. calleryana* (Stimart and Harbage, 1989). This suggests that some adaptive changes occur in the cultures before the onset of rapid shoot proliferation.

Data in Table 2 show that there was considerable increase in number of shoots per culture with an increase in BA concentration up to 2. 00 mg per litre, but a further increase in BA concentration reduced the number of shoots per culture. The highest number of shoots (11.24 shoots per culture) was obtained with 2.00 mg per litre and it was followed by 1.5 mg per litre BA (9.25 shoots per culture). Addition of 0.5 mg per litre IBA significantly increased the number of shoots per culture from 5.90 to 7.32. Cytokinins overcome the apical dominance of shoots and induce shoot proliferation and BA has been found to be the most effective cytokinin for stimulating axillary shoot proliferation, followed by kinetin and 2 iso pentyl adenine (Hu and Wang, 1983). A positive correlation between BA concentration and shoot multiplication up to certain level has been earlier reported in pear (Dwivedi and Bist, 1999 and Shibli et al., 1997). The increase in shoot proliferation following addition of low concentration of auxin in the MS medium has also been reported in pear (Dwivedi and Bist, 1999 and Bhojwani et al., 1984).

The interaction between BA and IBA was non significant. Although, increasing BA concentration in the culture medium, enhanced shoot proliferation yet shoot length decreased (Table 2). Higher BA concentration resulted in the shortening of shoots and the leaves became narrow and did not expand. Reduction in length of proliferated shoots by higher (2.0 and 2.5 mg per litre) BA caused difficulty in handling of shoots during shoot multiplication and rooting. Supplementation of shoot multiplication media with 0.5 mg per litre IBA increased shoot length. One of the possible roles of auxin during shoot proliferation is to nullify the suppressive effects of higher cytokinin concentration on axillary shoot elongation and restore normal shoot growth (Lundergan and Janick, 1980). Moreover, a plant species requires a specific auxin to cytokinin ratio for proper shoot proliferation and growth.

The increase in shoot length following addition of auxin in the shoot multiplication media may be due to

Table 2 Effect of growth regulator levels (mg l^{-1}) on the number of shoots produced per culture and shoot length (cm) during shoot multiplication

IBA	Number of shoots produced per culture			Shoot length (cm)		
BA	0.0	0.5	Mean	0.0	0.5	Mean
0.0	1.49	1.81	1.65	2.23	2.86	2.54
0.5	2.75	4.21	3.48	2.84	3.83	3.34
1.0	5.25	7.24	6.25	2.62	3.63	3.13
1.5	8.29	10.21	9.25	2.26	3.21	2.73
2.0	10.18	12.30	11.24	1.50	1.75	1.62
2.5	7.43	8.12	7.78	1.04	0.90	0.97
Mean	5.90	7.32		2.08	2.70	
LSD (0.05)		IBA (A) : (0.61) BA (B) : (1.06) A x B : NS			IBA (A) : (0.16) BA (B) : (0.28) A x B : (0.39)	

Table 3 Effect of supplementation of shoot proliferation media with adenine sulphate (mg l^{-1}) on the number of shoots per culture and shoot length (cm)

Adenine	Number of shoots per culture				Shoot length (cm)			
BA+IBA	0.0	0.5	1.0	Mean	0.0	0.5	1.0	Mean
1.0 + 0.5	7.24	8.07	6.24	7.18	3.63	2.75	1.86	2.75
1.5 + 0.5	10.21	11.12	10.13	10.49	3.21	2.10	1.45	2.25
2.0 + 0.5	12.30	12.96	11.21	12.16	1.25	1.21	1.10	1.19
Mean	9.91	10.72	9.19		2.69	2.02	1.47	
LSD (0.05)	Adenine (A) : NS BA + IBA (B) : (1.89) A x B : NS				Adenine (A) : (0.79) BA + IBA (B) : (0.81) A x B : NS			

nullification of suppressive effects of high cytokinin concentration. Shoot length was controlled by cytokinin and by lowering the cytokinin levels, shoots of desirable length have been obtained in *Pyrus calleryana* (Stimart and Harbage, 1989). Though, the highest number of shoots (11.24) were obtained with 2.0 mg per litre BA, but this BA level did not produce shoots of desirable length (>2.0 cm). BA (1.5 mg per litre) + IBA (0.5 mg per litre) was the best growth regulator combination for shoot multiplication in wild pear as it produced sufficient number of shoots (10.21 shoots per culture) having desirable shoot length.

The effect of adenine sulphate on shoot multiplication of *Pyrus* genotypes on MS medium fortified with BA (1.0-2.0 mg per litre) and IBA (0.5 mg per litre) was studied and the results are presented in Table 3. The data reveal that there was no significant effect of adenine sulphate on the number of shoots per culture (Table 3). Addition of adenine sulphate, significantly reduced the shoot length (Table 3). Shoot length is controlled by the level of cytokinin (Stimart and Harbage 1989). The decrease in shoot length may be due to the synergistic effect of the two different types of cytokinins (BA and adenine sulphate) used in the shoot multiplication media (Bowman 1994 and Baruah et al., 1995).

The effect of different nutrient media composition on shoot proliferation and shoot length is presented in Table 4. The media used were fortified with BA (1.5 mg per litre) and IBA (0.5 mg per litre). The highest number of shoots per culture (11.20) was obtained with Woody Plant Medium (WPM), followed by MS medium (10.21). The highest shoot length (3.21 cm) was observed with MS Medium and it was on a par with WPM. The MS and WPM media were superior MMS and ½ MS media, as they resulted in higher shoot number per culture. In the present investigations, reduction of nitrogen to one fourth of MS medium (MMS) and fifty percent reduction in al the MS salts (½ MS) did not enhance the proliferation rate (Table 4). George (1993) has cited a number of examples where reduction in nitrogen content or adjustment in nitrate to ammonium ratio of MS medium improved the *in vitro* shoot multiplication. However, in the present investigations, reduction of nitrogen to one fourth of MS medium (MMS) and fifty percent reduction in al the MS salts (½ MS) did not enhance the proliferation rate. The requirement of nutrient media have been shown to change with species, cultivar or even among different clones of a cultivar (Berardi et al., 1993). Banno et al., (1988) have also reported the superiority of WPM over ½ MS and ¼ MS media in *P. pyrifolia*.

Conclusions

There was no information regarding the micropropagation of 'wild pear' *Pyrus pyrifolia* (Burm F.). In the present study, explant establishment and shoot multiplication technique was standardized for 'wild pear'. Nodal segments explants proved to be better than shoot tips for initiating micropropagation of wild pear. When the explants were collected during spring and winter seasons there was higher explant establishment lower loss of the cultures due to explant browning and endogenic contamination. The minimum explant establishment and maximum phenolic browning and contamination was observed during rainy season. Antibrowning agents were more useful during spring and winter season when the incidence of brown-

Table 4 Effect of different media compositons supplemented with BA (1.5 mgl-1) and IBA (0.5 mgl-1) on shoot multiplication and shoot length

	MS	WPM	MMS	½ MS	LSD (0.05)
Number of shoots per culture	10.21	11.20	7.19	4.13	0.95
Shoot length (cm)	3.21	2.75	2.12	1.93	0.80

ing was low, in comparison to rainy, summer and autumn seasons when the browning was comparatively high. BA (1.5 mg per litre) + IBA (0.5 mg per litre) was the best growth regulator combination for shoot multiplication as it produced sufficient number of shoots (10.21 shoots per culture) having desirable shoot length. There was no significant effect of adenine sulphate on the number of shoots per culture but it significantly reduced the shoot length. The highest number of shoots per culture was obtained with Woody Plant Medium (WPM), followed by MS medium.

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