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Full Length Research Paper

In vitro induction of multiple buds from cotyledonary nodes of Balsam pear (*Momordica charantia* L.)

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In this study, cotyledonary nodes of Changbai, Dabai and Youlv Balsam pear (*Momordica charantia* L.) were used for tissue culture to establish regeneration system, and the effects of different genotypes of Balsam pear, hormone combinations, seedling ages, dark period and $AgNO_3$ concentrations on the bud regeneration were compared. The results show that eight-day old seedling was the best for multiple buds regeneration. The combination of 6-benzylaminopurine (6-BA) and indole-3-butyric acid (IBA) had the best effect on the induction of multiple buds. The optimum medium for the induction of multiple buds in Balsam pear was Murashige and Skoog (MS) medium supplemented with 2.5 mgL^{-1} of 6-BA and 0.1 mgL^{-1} of IBA. Inducing frequency varied with the genotypes, of which Changbai showed highest percentage of induction. One week dark treatment could increase the rate of shoot regeneration from 76.91 to 80.91%. The addition of $AgNO_3$ which varied from 1 to 6 mg L^{-1} in the medium could not help to stimulated shoot regeneration.

Key words: Balsam pear (*Momordica charantia* L.), multiple buds, tissue culture, regeneration.

INTRODUCTION

Balsam pear (*Momordica charantia* L.) is a weak climbing annual vine herbage plant belonging to the family, Cucurbitaceae. It is an important horticultural crop in subtropical and tropical regions, which is now widely cultivated throughout the world (Yamaguchi, 1983). Balsam pear originated in Asia and in the tropical areas of Africa. In the Amazon of South America, it is commonly consumed as food yielding and medicinal plant.

As an important and valuable vegetable, the fruit of the Balsam pear contains high amounts of glycosides, saponins, alkaloids, resins, phenolic constituents, free acids, reducing sugars and fixed oil (Dhalla et al., 1961). Ambasta et al. (1986) found out that the active ingredient of Balsam pear has good effects such as anti-rheumatic, anti-diabetic and carminative effects. It also has good effect on leprosy and could be used for bleeding piles, snake bites, urinary complaints, scorpion sting and as an antiseptic. In addition, some reports have also shown that it has significant anti-tumor growth and anti-HIV effects (Okabe et al., 1980; Lee-Huang et al., 1990; Lee-Huang et al., 1995).

There are many reports about the nutrition and medicinal value of Balsam pear, but the reports on *in vitro* regeneration of Balsam pear are few and of poor repeatability. We took the cotyledonary nodes of Balsam pear as explants and studied the influence of the elements, hormone combinations, seedling ages, dark period and $AgNO_3$ concentrations on the regeneration of adventitious buds. The paper is designed to develop an efficient protocol for tissue culture of Balsam pear and to establish an efficient, reproducible and fast plant regeneration system.

MATERIALS AND METHODS

The materials are the seeds of Changbai, Youlv and Dabai Balsam pear. The three cultivars are suitably popularized and utilized in the southern region of China. The seeds of Balsam pear were surface sterilized with 75% (w/v) of alcohol for 35 s. The seeds were then disinfected with 0.1% (w/v) of mercuric chloride solution for five minutes, after which the seeds were rinsed four to five times in

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Abbreviations: 6-BA, 6-Benzylaminopurine; I**BA,** indole-3-butyric acid; **MS,** Murashige and Skoog medium.

Figure 1. Germinated seedling.

autoclaved distilled water to remove traces of the mercuric chloride ($HgCl_2$) under a laminar air flow cabinet. Next, the seeds were placed on hormone free MS medium. The seeds started germinating within four days of inoculation and subsequently developed into complete plantlets within eight to 10 days (Figure 1).

Induction and elongation of multiple buds

The cotyledonary nodes of the seedlings at various ages from six to 11 days after germination were cultured on a Murashige and Skoog (MS) medium containing different concentrations and combinations of indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and 6-BA (1.0 to 3.0 mgL^{-1} of 6-BA; 0.05 to 0.2 mgL^{-1} IBA, IAA, NAA). For the preparation of explants, the terminal bud and lateral bud of the seedlings were excised; one of the cotyledons was also cut. The lower sides of the cotyledonary nodes which have 2 mm hypocotyls were implanted in a 30 ml medium dish.

Effect of genotype

The three varieties of Balsam pear cotyledonary nodes which had optimum seedling age were cultured on MS supplemented with suitable concentrations and combinations of cytokinins and auxins.

Effect of dark period

The best genotype of Balsam pear cotyledonary nodes which had optimum seedling age was cultured on the optimal medium and then the culture media were cultured in darkness for one, two and three weeks before putting them in the light.

Effect of $AgNO_3$

The best genotype of Balsam pear cotyledonary nodes which had optimum seedling age was implanted in the optimal medium supplement with different concentrations of $AgNO_3$ (1.0 to 6.0 mgL^{-1} of $AgNO_3$).

Rooting and transplanting

The vigorous shoots which have 1.5 cm length were transferred to a half strength MS medium supplemented with 0.2, 0.4 and 0.6 mgL^{-1} IBA for two to three weeks. The vigorous buds of the regenerated plants were transplanted and cultured in a pot until bloom and fruiting.

Data analysis

All the cultures were incubated at 25°C under cool-white fluorescent (50 µmol $m^{-2}s^{-1}$) lights with a 16 h photoperiod. After five weeks, the effect of the different genotypes of Balsam pear, hormone combinations, seedling ages, dark period and $AgNO_3$ concentrations on the induction of multiple buds was studied.

Data on the number of buds per culture and shoot regeneration percentage were recorded after five weeks. Then data on rooting days, rooting percentage, number of roots per culture and average length of roots were recorded. An analysis of variance (ANOVA)

Table 1. Multiple buds induction via cotyledonary nodes explants of *Momordica charantia* L at different seedling ages.

Seedling age (days)	Explants number	Buds of explants number	Induction rate of explant (%)
6	67	41	61.59[b]
8	65	51	78.63[a]
10	70	43	61.35[b]

Cotyledonary nodes explants cultured on MS medium with 2.5 mgL^{-1} 6-BA were evaluated after 5 weeks of culture. Values in a column followed by a common letter are significantly different at the 5% level (Duncan's multiple range test).

Table 2. Multiple buds induction via cotyledonary nodes of *Momordica charantia* L. at different genotypes.

Genotype	Average of multiple bud	Induction rate of explant (%)
Changbai	4.38[a]	78.79[a]
Dabai	3.38[b]	67.74[b]
Youlv	3.52[b]	80.65[a]

Cotyledonary nodes explants cultured on MS medium with 2.5 mgL-1 6-BA and 0.1 mgL-1 IBA were evaluated after 5 weeks of culture. Values in a column followed by a common letter are significantly different at the 5% level (Duncan's multiple range test). MS, Murashige and Skoog medium; IBA, indole-3-butyric acid; IAA, indole-3-acetic acid.

which was done using Duncan's multiple range test with a 95% confidence interval ($P<0.05$) was used to compare the means of all treatments.

RESULTS AND DISCUSSION

Effect of seedling age on induction of multiple buds

Seedling age is reported to be an important factor for obtaining high frequency adventitious shoot regeneration and the research results are diverse (Sultana et al., 2003; Wang et al., 2008; Zhang et al., 2008; Ntui et al., 2009). The seedling stage determines the physiological state of explants. Different physiological states of explants obviously, affect shoots regeneration capacity of explants (Lin et al., 2006). Therefore, the seedling stage is a key success factor of *in vitro* Balsam pear regeneration. Table 1 shows that there are significant differences in the induction rate of the different pear seedling ages. Shoot regeneration frequency in eight-day old seedlings is higher than in six- and 10-day old seedlings. The induction rate is 78.63%. In addition, the induction rate of multiple buds is high and the regenerated seedlings are vigorous when the seedling age is eight-day-old; this is consistent with Li et al. (2007).

Effect of genotype on induction of multiple buds

Many experimental results demonstrate the effect of genotype in the regeneration capacity of other plants in the Cucurbitaceae family (Wehner et al., 1981; Ntui et al., 2009). In Balsam pear tissue culture, genotype also has a considerable impact on the organogenic response (Wang et al., 2008; Al Munsu et al., 2009). From Table 2, we can conclude that, there are significant differences in the induction rate of the three Balsam pear cultivars in the experiment conducted. The induction rate of Youlv Balsam pear is highest (up to 80.65%), followed by the induction rate of Changbai Balsam pear (78.79%). The induction rate of Dabai Balsam pear was found to be the lowest (7.74%). However, the average multiple buds of Changbai were greater than that of the other Balsam pear cultivars. We also found that buds of "Changbai" Balsam pear are vigorous and easy to grow and it is the optimum genotype in the three varieties of Balsam pear.

Effect of hormone types and proportion on induction of multiple buds

The dedifferentiation and redifferentiation of plant cells is highly affected by the different plant hormone types and proportion, especially the proportion of auxins and cytokinins. Although some progress in regeneration improvement of Balsam pear has been made, the studies on the various hormones and proportion are diverse (Sultana et al., 2003; Agarwal et al., 2004; Song and Gao, 2006; Wang et al., 2008; Al Munsu et al., 2009). The cotyledonary nodes of Balsam pear are cultured on a medium (Table 3) and they begin to turn green and swell after two to three days. Callus appears at the lower edges of the cotyledonary nodes and multiple buds also appear after a week. As can be seen in Table 3, multiple buds can be induced on all kinds of media. Auxins and cytokinins play a great role in promoting the induction of multiple buds, particularly the effect of 6-BA.

The average of multiple buds was highest (up to 4.42) when explants were cultured on MS medium supplemented with 2.0 mgL^{-1} 6-BA and 0.1 mg L^{-1} NAA, but the buds were too weak and yellowish; it was difficult to elongate them. The multiple buds were not only large

Table 3. Effects of different hormone combinations and concentrations on the induction of multiple buds.

6-BA mgL^{-1}	IBA mgL^{-1}	NAA mgL^{-1}	IAA mgL^{-1}	Total buds of explant	Average buds per explant	Induction rate of explant (%)
3.0	0.05			94.0	3.35^{cdefg}	80.99^{c}
2.5	0.05			92.0	3.05^{efgh}	80.97^{c}
2.0	0.05			99.0	3.19^{efgh}	79.51^{cde}
1.0	0.05			56.0	2.69^{h}	71.37^{h}
3.0	0.10			117.0	3.54^{cde}	80.36^{cd}
2.5	0.10			190.0	4.14^{ab}	90.26^{a}
2.0	0.10			91.0	3.49^{cdef}	77.64^{de}
1.0	0.10			89.0	2.98^{fgh}	72.55^{gh}
3.0		0.20		106.0	3.80^{bc}	79.34^{cde}
2.5		0.20		79.0	3.17^{efgh}	74.52^{fg}
2.0		0.20		58.0	3.29^{cdefg}	73.68^{gh}
3.0		0.10		74.0	3.50^{cdefg}	74.64^{fg}
2.5		0.10		98.0	3.06^{efgh}	80.50^{cd}
2.0		0.10		111.0	4.42^{a}	84.58^{b}
3.0			0.10	68.0	3.26^{defg}	80.19^{cd}
2.5			0.10	86.0	3.76^{bcd}	81.83^{c}
2.0			0.10	82.0	2.92^{gh}	76.96^{ef}

Cotyledonary nodes explants cultured on MS medium were evaluated after 5 weeks of culture. Values in a column followed by a common letter are significantly different at the 5% level (Duncan's multiple range test). MS, Murashige and Skoog medium; IBA, indole-3-butyric acid; NAA, naphthaleneacetic acid; IAA, indole-3-acetic acid.

Figure 2. Multiple shoot buds.

in number, but also vigorous when explants were cultured on MS medium supplemented with 2.5mg L^{-1} 6-BA and 0.1 mg L^{-1} IBA (Figure 2). In addition, the induction rate of these different hormone types and proportions of MS medium reached as high as 90.26%. So these different hormone types and proportions of MS medium were the optimum medium in the regeneration of Balsam pear. The phenomenon of crisped-leaf, expanded stem, and vitrify-

Figure 3. Elongation of multiple buds.

Table 4. Effects of dark treatment on the induction of multiple buds.

Dark period (days)	Induction rate of multiple bud (%)	Average of buds per explant
0	76.91[b]	4.01[a]
7	80.91[a]	4.21[a]
14	77.93[b]	4.11[a]
21	71.25[c]	3.47[c]

Cotyledonary nodes explants cultured on MS medium with 2.5 mgL^{-1} 6-BA and 0.1 mgL^{-1} IBA were evaluated after 5 weeks of culture. Values in a column followed by a common letter are significantly different at the 5% level (Duncan's multiple range test). MS, Murashige and Skoog medium; IBA, indole-3-butyric acid; IAA, indole-3-acetic acid.

cation occurred commonly when the MS medium was supplemented with high concentration of 6-BA. However, a low concentration of 6-BA (1.0 mgL^{-1}) was good for the elongation of buds when they were moved to the MS medium supplemented with it (Figure 3). Different hormone types and proportions had a great influence on the induction of multiple buds in this experiment. At present, NAA and 6-BA are commonly used in Balsam pear tissue culture (Sultana et al., 2003; Agarwal et al., 2004; Wang et al., 2008; Al Munsu et al., 2009). However, we found out that the effect of IBA was better than NAA and IAA in this experiment. This phenomenon is probably dependent on the genotypes of the Balsam pear tissue culture.

Effect of different dark period treatment on induction of multiple buds

The cotyledonary nodes of Balsam pear which are eight-day old were implanted in the MS medium supplemented with 2.5 mgL^{-1} 6-BA and 0.1 mgL^{-1} IBA with different dark period treatments. After five weeks, the statistical results (Table 4) showed that the average number of multiple buds was obviously high in contrast to the population without dark treatment. The best dark treatment dose was

Table 5. Effects of different $AgNO_3$ concentrations on the induction of multiple buds.

$AgNO_3$ (mgL^{-1})	Induction rate of multiple bud (%)	Average of multiple bud
0	74.99[a]	3.75[a]
1	55.70[b]	2.64[b]
2	42.43[c]	1.47[c]
3	29.86[d]	0.86[d]
4	9.87[e]	0.32[e]
5	0[f]	0[f]
6	0[f]	0[f]

Cotyledonary nodes explants cultured on MS medium with 2.5 mgL^{-1} 6-BA and 0.1 mgL^{-1} IBA were evaluated after 5 weeks of culture. Values in a column followed by a common letter are significantly different at the 5% level (Duncan's multiple range test). MS, Murashige and Skoog medium; IBA, indole-3-butyric acid; IAA, indole-3-acetic acid.

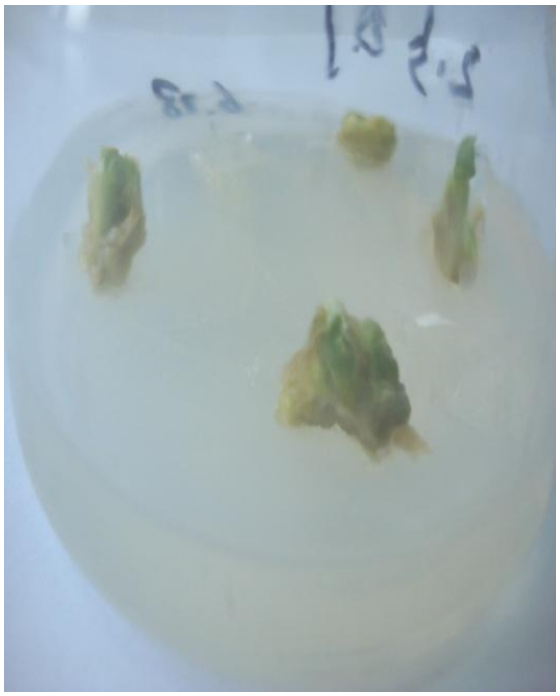

Figure 4. Effect of AgNO3 on explants.

seven days and the induction rate reached up to 80.91%. However, when the dark periods increased to 21 days, the average of multiple buds was lower than that of the cotyledonary nodes without dark treatment. In this experiment, dark period treatment had a significant improving effect on the induction of multiple buds. Some studies, however, showed that dark period treatment could slow the inactivation of IAA in Balsam pear which made them to maintain the concentration of IAA at a certain level (Li et al., 2007).

Effect of different $AgNO_3$ concentrations on induction of multiple bud

The cotyledonary nodes of eight-day old Balsam pear were cultured on MS medium supplemented with 2.5 mgL^{-1} 6-BA and 0.1 mgL^{-1} IBA with different concentrations of $AgNO_3$ (Table 5). The induction of multiple buds was inhibited obviously by $AgNO_3$. The cotyledonary nodes of explants were neither induced shoots nor induced roots, so those cotyledonary nodes appeared etiolating and dead as the culture time progressed, and the $AgNO_3$ concentration increased to 5 mgL^{-1} (Figure 4).

Rooting

Rooting of regenerated buds is important for establishing tissue culture derived from plants. It is generally accepted that the high rate of auxin / cytokinin helped to regenerate roots. On the contrary, the low rate of auxin / cytokinin

Figure 5. Rooting on multiple shoots.

Table 6. Effects of different IBA concentrations on the induction of rooting.

Medium	IBA mgL^{-1}	Average of root per seedling	Average length of the root (cm)
1/2 MS	0.2	6.67^{b}	2.23^{b}
1/2 MS	0.4	8.33^{a}	2.77^{a}
1/2 MS	0.6	5.61^{c}	2.15^{b}

Values in a column followed by a common letter are significantly different at the 5% level (Duncan's multiple range test). IBA, Indole-3-butyric acid.

helped to regenerate buds. When the regenerated buds reached a height of 2 to 3 cm, the individual buds were carefully excised and transferred to different rooting media for proper rooting. In this experiment, IBA was effectively used as a root inducing hormone. This is in agreement with other reports (Minocha et al., 1987). The best root development (Figure 5) was obtained in the rooting medium containing a half strength of MS medium with 0.4 mg/L IBA (Table 6) and the rooting rate reached 80 to 90% (Figure 6). About two weeks was required to get healthy root formation. It was observed that singly, IBA was effective in the induction of root in different plants like *Momordica charantia* L (Agarwal *et al*., 2004), *Cuminum cyminum* L. (Azza et al., 2001) and *Limonium altaica* L.(Jeong et al., 2001).

Transplant of seedlings

The success of seedlings transplant was dependent on the integrity of roots. The regenerated plantlets were cultured for three to four days in natural light. Then the culture bottles were kept open to induce stress on the seedlings for two days. It is necessary to maintain the required moisture content during this period. The rooting seedlings were cleaned by removing them from the medium and transferring them to a nutrient rich soil which consisted of turfy soil and vermiculite (1:1, V:V) (Figure 7).

Figure 6. Rooting on multiple shoots.

Figure 7. Regenerated and hardened plantlet.

Figure 8. Regenerated and hardened plantlet.

The survival rate of seedlings was 90% (Figure 8).

Conclusion

Although some progress has been made in the regeneration of Balsam pear, there is much work to be done in this direction. Due to the fact that the study of the breeding biotechnology of Balsam pear has become relatively few and slow, it is difficult to establish the regeneration system of *in vitro* culture of Balsam pear. Some researchers found out that the vitrification, browning, yellowing and low frequency of induction phenomenon are serious in the tissue culture of Balsam pear. Thus, making further efforts to optimize the regeneration system of Balsam pear to obtain a high-effective and genotype-independent regeneration system is still an important goal in Balsam pear.

The establishment of an efficient and reproducible regeneration system is necessary for most biotechnology-mediated crop improvement. In this experiment, we found out that multiple buds can be induced effectively by cotyledonary nodes explants of Balsam pear. This was consistent with the earlier reports (Murashige and Skoog, 1962; Cheng and Voqui, 1977; Christopher and Rajam, 1994). Different seedling age and site of cotyledonary nodes had different effects on multiple buds regeneration. The eight-day old seedling was the best for multiple buds. The different hormone combinations and concentration had significant effects on the induction of multiple buds. The combination of 6-BA and IBA had the best effect. The optimum medium for the induction of multiple buds in Balsam pear was MS medium supplemented with 2.5 mgL^{-1} 6-BA and 0.1 mgL^{-1} IBA. The optimum genotype and dark treatment periods could also be good for increasing the rate of shoot regeneration effectively. We demonstrated an efficient plant regeneration system through organogenesis using proximal cotyledon nodes explants excised from seedlings after *in vitro* germination. We believe that this efficient shoot regeneration system for a diploid plant such as Balsam pear will contribute to the production of transgenic plants as well as be a practical tool for Balsam pear improvement and the study of its physiology.

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