African Journal of Biotechnology Vol. 10(34), pp. 6447-6455, 11 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1644
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Establishment of high effective regeneration and propagation system for ornamental crabapple (*Malus* spp.)

Jin Kaina[1], Zhang Jie[1,2], Liu Junli[1], Geng Hui[1], Shen Hongxiang[1,2] and Yao Yuncong[1,2*]

[1]Plant Science and Technology College, Beijing University of Agriculture, Beijing 102206, China.
[2]Beijing Key Laboratory of New Technology in Agricultural Application, College of Plant Science and Technology, Beijing University of Agriculture, Beijing 102206, China.

Accepted 6 April, 2011

In order to establish efficient regeneration system for ornamental tissue culture, we used *Malus* spp. 'Indian Magic as the experimental materials and investigated the effects of disinfection and anti-browning agents, culture mediums and hormones proportion on differentiation, multiplication, callus induction and rooting, and also the effects of culture substrates on growth of transplants in greenhouse. The results showed that, the selection of stem tips without scale as the tissue culture material and the employment of $HgCl_2$ 5+3 min two-step method for stem tip disinfection could reduce contamination rate and the addition of 2.0 g/l PVP (polyvinylpyrrolidone) could effectively prevent the browning of culture medium and explants. When the three kinds of culture medium were fixed [Murshige and Skoog (MS) containing 1.0 mg/l 6-BA (6-benzyladenine) plus 0.01 mg/l NAA (a-naphthaleneacetic acid), MS containing mg/l 6-BA plus 0.2 mg/l NAA and 1/2 MS containing 0.2 mg/l NAA] on inducing differentiation, multiplication and rooting, the differentiation rate, multiplication coefficient and rooting rate increased to 81.2, 6.13 and 100%, respectively. At the same time, leaves callus induction rate reached 100% on the medium of MS containing 1.5 mg/l 6-BA plus 1.0 mg/l 2,4-D(2,4-dichlorophexoxyace-tic acid) and adventitious shoots were directly regenerated from leaves on MS containing 1 mg/l 6-BA plus 0.1 mg/l NAA. The survival rate of the transplants was up to 95% and seedlings grew well after the transplant to the substrates containing vermiculite and perlite (1:1) in the greenhouse under relative humidity of 80 to 85%, temperature of 25 ± 2°C, light intensity of 1500 to 2000Lx and mist spraying condition. Based on the stated, we determined a technical framework of the leaves callus induction and regeneration for crabapple tissue culture, in which both the types and concentrations of hormones added in the medium played most important roles.

Key words: *Malus* spp., regeneration, callus induction, propagation, hormones.

INTRODUCTION

As one of *Rosaceae Malus* plants, crabapple has extra-ordinarily ornamental value and is often selected and used in the forestation and beautification such as courtyard, pavement and greenbelt due to its amicable shape of tree canopy, long flowering period, bright and diverse color of flowers, fruits and leaves, strong resistance and so on. Recently, more and more concerns have been shown on how to overcome the problems of low multiplication coefficient and seedling production caused by traditional breeding methods. As a result, with the development of tissue culture technology, researches on rapid propagation technology of perennial fruit trees go in depth; efficient regeneration and genetic

*Corresponding author. E-mail: yaoyc_20@bac.edu.cn.

Abbreviations: MS, Murashige and Skoog medium; **6-BA,** 6-benzylaminopurine; **NAA,** a-naphthaleneacetic acid; **IBA,** indole-3-butyric acid; **TDZ,** thidiazuron; **2,4-D,** 2,4-dichlorophexoxyace-tic acid; **PVP,** polyvinylpyrrolidone.

transformation system are improved gradually and the degree of marketing for tissue culture seedlings increases step by step. Meanwhile, the research of *Malus* plants tissue culture is developing rapidly.

By now, many researchers have carried out a lot of useful discussions in selecting various materials (tissues and organs) of different varieties, screening culture medium, setting culture conditions and exercising seedlings outdoor. Zhang showed that, multiplication coefficient reached up to 7 on the medium of MS containing 0.7 mg/l 6-BA plus 0.3 mg/l NAA and rooting rate was up to 95% on the medium of ½ MS containing 0.3 mg/l IBA(Indole-3-butyricacid) for *Malus halliana* (Zhang et al., 2007). In terms of leaves callus induction, it was found that the leaves of *Malus prunifolia* started generating callus after 14 days in darkness and callus induction rate reached 100% after 30 days on MS containing 5 mg/l 6-BA plus 0.1 mg/l 2,4-D (Zhang et al., 1992). Liu used *Malus domestica* 'Starkrimson' as the material to establish a high efficient regeneration system and found that, the optimal explants for regeneration was leaf given the optimal culture medium of MS containing 1.0 mg/l TDZ (thidiazuron) plus 0.3 mg/l NAA (Liu et al., 2007). However, it should be noted that due to differences in cultivars and plant materials, technology steps such as preventing the explants contamination, selecting culture medium, regulating hormone proportion and exercising seedlings outdoor have been the difficulties in the establishment of regeneration system for *Malus* plant, especially for crabapple.

It is well known that successful transformation of woody fruit trees relies on a good regeneration system, but an efficient regeneration system has not been previously reported for *Malus* 'Indian magic'. Therefore, in this study we used *Malus* 'Indian magic' as the material and set up series of treatments on anti-polluting, anti-browning, culture medium types, hormone proportions and cultured substrates for transplants. Through the treatments, we tried to promote callus induction, increase seedling propagation coefficient and improve their growth status, so as to establish a higher efficient system of tissue culture regeneration and propagation for crabapple.

MATERIALS AND METHODS

Plant materials

The experimental materials were from one-year-old branches of *Malus* 'Indian magic' plants at the age of six, growing vigorously with no pest or disease damage, from Crabapple Germplasm Resources Garden of Beijing University of Agriculture, Changping District in Beijing.

Treatments design and test methods

Materials pretreatment

One-year-old branches were taken to the laboratory in spring before germination. The base of the branches were immersed in water of 20 to 25°C until the axillary buds germinated, then the branches were cut into 2 to 3 cm stem segments with single-bud. These stem segments and stem tip sections were rinsed with tap water for 2 h, soaked with 75% alcoholic solution for 30 s, washed with sterile water for 3 times, disinfected with 0.15% $HgCl_2$ for 8 min, and flushed with sterile water for 3 times in sequence. We took them on the ultraclean table and peeled scales at growing points. Then, 2.0 to 3.0 mm long stem tips and stem segments were separately inoculated on culture mediums with sucrose concentration of 30 g L^{-1}, agar concentration of 6.0 g L^{-1} and cultured temperature of 24 to 26°C, light intensity of 1500 to 2000Lx and 12 to 16 h light a day.

Explants disinfection and anti-browning treatments

Screening of explants types: Stem sections and stem tips from *Malus* crabapple 'Indian magic' were inoculated into the culture medium of MS containing 0.5 mg·L^{-1} 6-BA plus 0.05 mg·L^{-1} NAA. The germination rate of explants was investigated, respectively.

Disinfection of stem tips and test of contamination rate: Three different disinfection patterns of using $HgCl_2$ were set: (1) T8, disinfection of stem tips with scale for 8 min; (2) T4+4, disinfection of stem tips with scale for 4 min, and then disinfection without scale for another 4 min and (3) T5+3, disinfection of stem tips with scale for 5 min, and then disinfection without scale for another 3 min. The contamination rate of inoculated stem tissues was tested after 10 days. Germination index was indicated as follows: no stem tips germinated was marked with "0", 30% was marked with "1", 50% was marked with "2" and 70% was marked with "3".

As for the anti-browning treatments of stem tips and test of browning rate, 4 treatments were set: addition of 0, 0.5, 1.0, 1.5, 2.0 g/l PVP to the culture medium of MS containing 6-BA 0.5 mg L^{-1} plus NAA 0.05 mg L^{-1}, respectively (Zhou et al., 2000); the browning rate of the inoculated stem tissues was tested after 10 days.

Selection of culture medium and hormones treatments

Screening of culture medium for differentiation: MS, 1/2MS, ¼ MS (containing 1.0 mg/l 6-BA and 0.02 mg/l NAA) mediums for stem tips tissue culture were set and the germination rate of stem tips was investigated after 30 days.

Hormones treatments: In MS medium, 3 concentrations of different hormones were added as treatments: (1) 0.5 mg/l 6-BA and 0.01 mg/l NAA; (2) 1.0 mg/l 6-BA and 0.01 mg/l NAA; (3) 1.0 mg/l 6-BA and 0.02 mg/l NAA. The germination rate after 30 days and the differentiation rate of stem tips after 50 days were investigated, respectively. Differentiation grades were defined as follows; when explants differentiated and grew well, 2 points were given as the first grade; when explants tended to differentiate and grew slowly, 1 point was given as the second grade; when explants tended to die or had died, 0 point was given as the third grade. Differentiation index equals $\sum$ (point of each grade × number of each grade) / (point of first grade × total number).

Screening of culture medium for multiplication: Five treatments were set (Table 1) and the growth status of the cultured seedlings was observed after 30 days.

Screening of the culture medium for rooting: Six treatments were set (Table 2) and the growth status of the cultured seedlings was observed after 20 days. Nine treatments for callus induction were set as Table 3. The leaves explants from the cultured seedlings were cultured in darkness for 10 days and with light for 20 days on these mediums. Leaves callus induction frequency was investigated

Table 1. Treatments for multiplication.

Treatment	6-BA (mg/l)	TDZ (mg/l)	NAA (mg/l)
MBN1	0.5		0.2
MBN2	1.0		0.2
MTN3		0.01	0.2
MTN4		0.05	0.2
MTN5		0.1	0.2

Table 2. Treatments for rooting.

Treatment	Basic medium	6-BA (mg/l)	NAA (mg/l)	IBA (mg/l)
Tr1	MS			
Tr2	MS	0.5	0.1	
Tr3	1/2 MS			
Tr4	1/2 MS	0.2		
Tr5	1/2 MS		0.5	
Tr6	1/2 MS			0.5

Table 3. Treatments for callus induction.

Treatment	6-BA (mg/l)	NAA (mg/l)	2,4-D (mg/l)
T1	1		
T2	1		0.5
T3	1.5		1
T4	1	0.1	
T5	1.5	0.1	0.5
T6	0.5	0.1	1
T7	1.5	0.2	
T8	0.5	0.2	0.5
T9	1	0.2	1

by the number of leaves emerging callus/number of total leaves.

Screening of substrates for transplants

When the tissue culture seedlings grew up to 5 to 6 cm high and roots elongated to 3 to 5 cm; we set (1) vermiculite + peat + sand (1:1:1) and (2) vermiculite + perlite (1:1) as the treatments, opened the caps of culture flasks, used warm water to wash the seedlings after 3 days, then transplanted the seedlings into sterile nutritional bowls of two treatments watered sufficiently. At the relative humidity of 80 to 85%, temperature of 25 ± 2°C and light intensity of 1500 to 2000 Lx, the potted seedlings were placed in greenhouse and were covered by polyethylene film with mist spray once a day (Wei et al., 2004). Investigation of survival rate and growth status of the transplanted seedlings was conducted after 20 days.

Data analysis

The experiment was designed as a randomized block with three replications. The samples of each treatment were 30. Data statistics were done by Excel and SPPS 12.0 version.

RESULTS

Contamination and browning condition of cultured stem tips from *Malus* 'Indian magic' under different treatments

Contamination and germination rate of different explants

As for the tissue culture, it was most important to choose suitable tissues for the germination, differentiation and multiplication of explants. Usually, contamination and germination rate of different explants are different, even in the same variety and species. It can be seen from Table 4 that, when the stem tips and stem segments of *Malus* 'Indian magic' were used as explants cultured in MS containing 0.5 mg/l 6-BA plus 0.05 mg/l NAA, the contamination rate of stem tips culture was significantly lower than that of the stem segments culture and the germination rate of cultured stem tips was significantly

Table 4. Contamination rate and germination rate of different kinds of explants.

Explant	Contamination rate (%)	Germination rate (%)
stem segments	97.00^{Aa}	0^{Bb}
stem tips	40.00^{Bb}	33.33^{Aa}

Capital and case letters indicate significance at $p< 0.01$ and $p< 0.05$ by Duncan's significant test.

Table 5. Effect of different time of $HgCl_2$ disinfection on the contamination rate of cultured stem tips.

$HgCl_2$ treatment	Contamination rate	Contamination of medium[1]	Germination index of stem tip
T8	43.3 ^{Aa}	++	0
T4+4	33.3 ^{Bb}	+	2
T5+3	21.7 ^{Cc}	+	3

[1]contaminated surface was less than half of the medium surface marks +, and contaminated surface was more than half of the medium surface marks ++. Capital and case letters indicate significance at $p< 0.01$ and $p< 0.05$ by Duncan's significant test.

Table 6. Browning rate of the cultured stem tips from *Malus* 'Indian magic' by treating with different PVP concentrations.

PVP concentration (g/l)	Browning rate (%)	Medium browning[1]	Germination index of stem tip
0	100.0^{Aa}	××	0
0.5	73.3^{Bb}	××	1
1.0	53.3^{Cc}	×	2
2.0	16.7^{Dd}	×	3

[1]Browning area was less than half of the medium surface marks "×" and browning area was more than half of the medium surface marks "××".Capital and case letters indicate significance at $p< 0.01$ and $p< 0.05$ by Duncan's significant test.

higher than that of the stem segments.

Contamination rate and growth status of stem tips under treatments of $HgCl_2$

Disinfection with $HgCl_2$ is a common method and an important step in tissue culture. Table 5 shows that, there were significant differences for the contamination rate of stem tips among the different disinfection treatments of $HgCl_2$. In T5+3 treatment, the stem tips had the lowest contamination rate and most stem tips germinated with lightly polluted culture medium. In T4+4 treatment, half of the cultured stem tips germinated with light pollution of the culture medium. In T8 treatment, contamination rate of the cultured stem tips was the highest, reaching 43.3%, with severe pollution of culture medium.

Browning rate of stem tips by different concentrations of PVP treatments

During culturing of *Malus* crabapple, when stem tips were incised, browning often occurred, resulting in decreased survival rate of the explants. To restrain browning, different concentrations of PVP, an anti-browning agent was added in the culture mediums. Results showed that, explants browning rate reached 100% in culture mediums without PVP (Table 6). When PVP concentration increased, the browning rate of the stem tips and browning area presented in the culture medium decreased and at the same time, germination index of explants increased. When 2.0 g/l PVP was added to the culture mediums, the browning rate of the stem tips was brought down to 16.7%, and the browning area presented in the culture medium shrunk; less than half of the medium surface. Consequently, most of the stem tips germinated and grew better.

Effects of different types of culture mediums as well as hormone concentrations on stem tips differentiation, multiplication and rooting from *Malus* 'Indian magic'

Effects of both culture medium types and hormone concentrations on differentiation of stem tips

The types of culture medium and hormone concentrations play key roles in the differentiation, proliferation and rooting of explants. For different varieties and even tissues and organs of plants, there are suitable culture medium and hormone concentrations for explants (Thorpe, 1983; Yeh-Jin et al., 2007; Casanova et al.,2008;

Table 7. Effect of both culture medium types and hormone concentrations on germination and differentiation of stem tips.

Treatment	Hormone concentration (mg/l)		Germination rate after 30 days (%)	Differentiation rate after 50 days (%)	Bud number of different grade			Differentiation index (%)
	6-BA	NAA			First	Second	Third	
MS	0.5	0.01	80.00^{Aa}	0^{Cc}	0	0	0	-
	1.0	0.01	80.00^{Aa}	81.20^{Aa}	0	0	0	-
	1.0	0.02	78.40^{Aa}	66.70^{Bb}	7	9	2	63.89^{Aa}
1/2MS	1.0	0.02	-	-	3	6	5	42.86^{Bb}
1/4MS	1.0	0.02	-	-	1	5	10	21.88^{Cc}

Capital and case letters indicate significance at $p< 0.01$ and $p< 0.05$ by Duncan's significant test.

Table 8. Effect of different culture mediums on multiplication of cultured explants.

Index	MBN1	MBN2	MTN 3	MTN 4	MTN 5
MC	3.37^{dD}	6.13b^{B}	4.60^{cC}	6.37^{aA}	1.10^{eE}
GS	Robust tender green	Very robust green	Robust tender green	Not robust part vitrification	Weak all vitrification

MC, Multiplication coefficient; GS, growth status of tissue cultured seedlings. Capital and case letters indicate significance at $p< 0.01$ and $p< 0.05$ by Duncan's significant test.

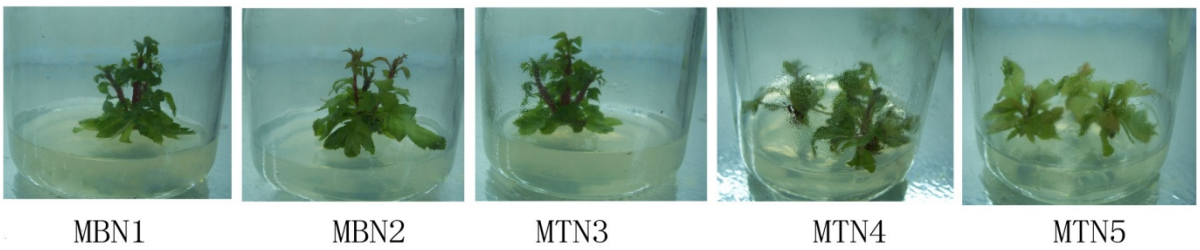

Figure 1. Growth status of the tissue cultured seedlings by different treatments. MBN1, MBN2, MTN3, MTN4 and MTN5 are same as Table 8.

Kakani et al., 2009). In order to screen suitable differentiation medium for stem tips from *Malus* 'Indian magic' plant, stem tips were inoculated on MS, ½ MS, ¼ MS medium containing 1.0 mg/l 6-BA, 0.02 mg/l NAA and 2.0 g/l PVP. It was found that, the differentiation index of stem tips among the three kinds of medium varied significantly. The differentiation index on the MS medium was the highest, reaching 63.89%; in comparison, the differentiation index of the other two kinds of culture medium were relatively low (Table 7).

Furthermore, after adding different hormone concentrations to the MS medium to investigate the effects on germination and differentiation of explants, we found no significant differences in the germination rates of the explants after 30 days, while the differentiation rates of the three treatments after 50 days showed significant differences among the different treatments of hormone concentrations. As shown in Table 4, the differentiation rate of explants on MS containing 0.5 mg/l 6-BA plus 0.01 mg/l NAA was almost 0, whereas, that on MS containing 1.0 mg/l 6-BA +0.01 mg/l NAA reached up to 81.2%.

Effects of hormone kinds and concentrations on multiplication of explants

In order to screen suitable medium for multiplication of explants from *Malus* 'Indian magic', we transferred the vigorous shoots from the differentiation medium to the multiplication medium with five treatments of different hormone kinds and concentrations. As shown in Table 8, with the increasing concentration of 6-BA in the mediums, the multiplication coefficient increased and with the increasing concentration of TDZ in the medium, the multiplication coefficient firstly increased and then decreased and seedlings were in poor growth status and tended to vitrify. The multiplication coefficient of the treatment of MBN2 was 6.13 and seedlings grew robustly (Figure 1); therefore, it was considered to be a suitable medium for multiplication of explants from *Malus* 'Indian

Table 9. Effects of different culture mediums and hormone concentrations on the rooting rate and root growth of cultured seedlings.

Treatment	Rooting rate (%)	Average root number	Average root length (cm)	Growth status of root
Tr1	95 ^{Bb}	11	5.8	Thick, long
Tr2	76 ^{Dd}	8	2.3	Thin, short ,callus in stem
Tr3	90 ^{Cc}	9	1.8	Thick ,short, callus in stem
Tr4	100 ^{Aa}	14	4.2	Thick, long
Tr5	67 ^{Ee}	6	1.7	Short, callus in root
Tr6	100 ^{Aa}	19	3.7	Small callus in stem

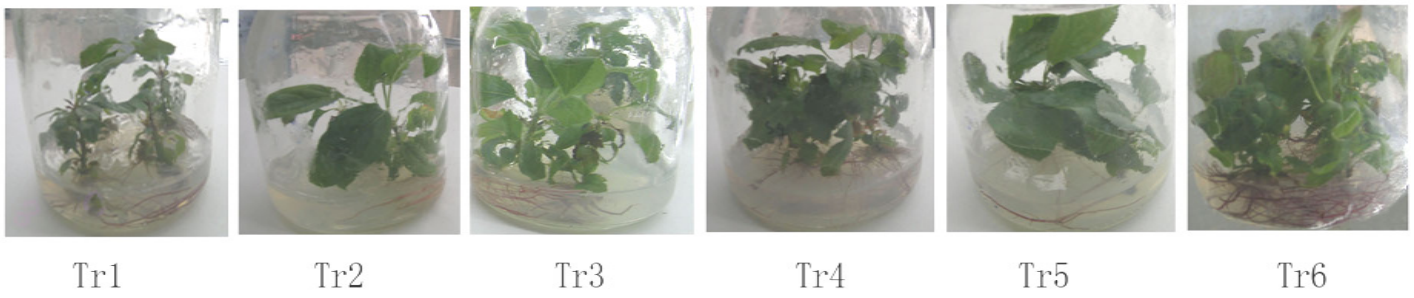

Figure 2. Root growth of seedlings cultured by different treatments.Tr1, Tr2, Tr3, Tr4, Tr5 and Tr6 are same as Table 9.

magic' plants.

Effects of different culture medium and hormone concentrations on the rooting rate and root growth for seedlings of Malus 'Indian magic'

After multiplication culture, the vigorous seedlings were transferred to different rooting mediums. We used six mediums with different medium types and hormone concentrations and investigated the rooting rate and root growth status to screen suitable rooting medium (Table 9; Figure 2). Results showed that, the rooting rate of Tr4 and Tr6 were up to 100% with long, large number of roots and only a small amount of callus in stem in T6, which was significantly higher than that in other treatments. The rooting rate of Tr2 and Tr5 were lower than that of others, with small number of short roots and also callus in stems and roots, respectively. The rooting rate of Tr3 was 90%, with short and thick roots and callus in the stems. The rooting rate of Tr1 was 95%, with thick roots. Thus, we concluded that Tr4 might be a suitable rooting medium for *Malus* 'Indian magic', Tr5 was good for induction of callus in root and Tr2 and Tr3 were suitable to induce callus in stem.

Effects of different hormone kinds and concentrations on leaf callus induction and regeneration of *Malus* 'Indian magic'

In this study, we set nine treatments of different hormone kinds and concentrations for leaf callus induction of *Malus* 'Indian magic' (Table 10). It was proved that, callus induction rate in T3 treatment was up to 100% and callus bulk was relatively large (Figure 3b); the callus induction rate in T2, T6 and T8 treatments were more than 90%; and in T4 and T7 treatments containing NAA but no 2,4-D, callus induction rates were about 50%. It was indicated that, effect of 2,4-D on callus induction was significantly greater than that of NAA. In addition, we found adventitious bud emerging on leaves in T4 (Figure 3a). It was suggested that, leaves of *Malus* 'Indian magic' could directly regenerate in T4 treatment though the regenerate rate was 33%.

Survival rate of transplanted tissue culture seedlings of *Malus* 'Indian magic' in different culture substrates

The high survival rate of the transplanted tissue culture seedlings is the key to getting plantlets. To ensure successful transplantation, vigorous seedlings with roots and without roots were transferred to the pots with two kinds of culture substrates, one containing vermiculite plus grass peat plus sand (1:1:1) and another containing vermiculite plus perlite (1:1). It can be seen from Table 11 that, the survival rate of seedlings growing on vermiculite plus perlite was higher; the survival rate of seedlings with roots was up to 95% with better growth status (Figure 3c) and the survival rate of those without roots was 70%. The survival rate of seedlings growing on vermiculite plus peat plus sand was low; the survival rate of seedlings with roots was 30%, while the survival rate of those

Table 10. Effect of different hormone concentrations on leaf callus induction of *Malus* 'Indian magic'.

Treatment	Callus (%)	Color	Zize[1]
T1	0 ^{De}	No	No
T2	97.03 ^{ABab}	White, pale yellow	Middle
T3	100 ^{Aa}	White, pale yellow	Big
T4	53.74 ^{Cd}	White, pale green	Small
T5	83.10 ^{ABbc}	White, pale yellow	Middle
T6	90.10 ^{ABbc}	White, pale yellow	Big
T7	48.51 ^{Cd}	White, pale green	Small
T8	90.02 ^{ABabc}	White, pale yellow	Big
T9	81.43 ^{Bc}	White, pale yellow	Middle

Note: 1, Small" means callus area of one leaf is less than 0.5 cm^2, middle" means callus area of one leaf is between 0.5 and 1 cm^2, big" means callus area of one leaf is more than 1 cm^2. Capital and case letters indicate significance at $p< 0.01$ and $p< 0.05$ by Duncan's significant test.

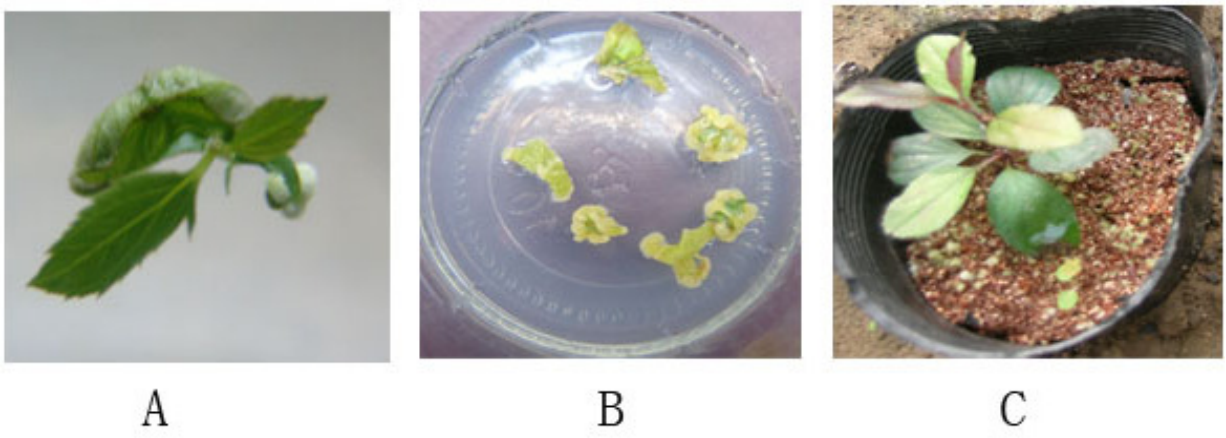

Figure 3. Propagation system for ornamental crabapple (*Malus* spp.). A, Adventitious bud induction directly from leaf; B, leaf callus; C, seedlings growth on substrate containing vermiculite and perlite (1:1).

Table 11. The survival rate and growth status of the cultured *Malus* 'Indian magic' seedlings after transplant.

Treatment	Type of tissue culture seedling	Survival rate (%)	Growth status of seedling
vermiculite + grass peat + sand (1:1:1)	Seedling with roots	30 ^{Cc}	weak
	Seedling without roots	10 ^{Dd}	weak
vermiculite+ perlite (1:1)	Seedling with roots	95 ^{Aa}	robust, fast growing
	Seedling without roots	70 ^{Bb}	weak, slow growing

without roots was only 10%.

DISCUSSION

Factors influence the establishment of regeneration system for crabapple

The establishment of plant regeneration system is an important basis for optimizing the preservation of germplasm resources, rapid breeding of plantlet and building of genetic transformation system in woody plants. Due to the diversity and complex genetic background of different plant species and cultural materials, it requires different pre-treatments, various medium types for differentiation, multiplication and rooting of explants, additional hormone kinds and concentrations, as well as culture environment conditions. As a result, the construction of regeneration system and genetic transformation system of woody plant make slow

progress (De Klerk et al., 1995; Kikkert et al., 1996; Han et al., 2009). In this study, we used *Malus* 'Indian magic' as the material to study some key factors for the establishment of efficient regeneration and induction system. Firstly, we investigated the effects of cultural materials, disinfection time and anti-browning treatments on explants. Secondly, effects of medium types, hormone kinds and concentrations on the differentiation, multiplication, rooting of explants and on the growth of seedlings from stem tips were determined. And at last, we probed the survival rate and growth status of the transplanted tissue culture seedlings in different culture substrates. Finally, we successfully constructed a regeneration system for explants from *Malus* 'Indian magic' plants. Under the culture conditions temperature of 24 to 26°C, light intensity of 1500 to 2000Lx and light of 12 to 16 h/day, the selection of stem tips without scale as the tissue culture material and the use of $HgCl_2$ 5+3 min two-step method for stem tips disinfection reduced contamination rate and the addition of 0.2 mg·L^{-1} PVP effectively prevented the browning of culture medium and explants. The employment of MS containing1.0 mg/l 6-BA plus 0.01 mg/l NAA, MS containing1.0 mg/l 6-BA plus 0.2 mg/l NAA and 1/2 MS containing 0.2 mg/l NAA as culture mediums significantly enhanced the differentiation rate, multiplication coefficient and rooting rate of the explants, respectively. The use of MS containing 1.5 mg/l 6-BA plus 1.0 mg/l 2,4-D as culture medium significantly increased the leaf callus induction rate. On the MS medium containing 1 mg/l 6-BA and 0.1 mg/l NAA, leaves could directly regenerate without callus induction. When well-grown seedlings were transplanted to the substrate of vermiculite + perlite (1:1), the survival rate of transplants was obviously higher and their growth status was better in the greenhouse at the relative humidity of 80 to 85%, temperature of 25 ± 2°C, light intensity of 1500 to 2000Lx and mist spraying conditions. This system provided not only a direct way of establishment of regeneration system and genetic transformation system for crabapple, but also technical support for resources preservation and propagation of other closely-allied woody plants.

There are many factors that can affect the construction of tissue culture regeneration of woody plants, including the genetic characteristics of plant varieties, properties of tissues and organs, culture mediums and hormone kinds and concentrations, environmental conditions and so on. For example, explants contamination and browning degree varied due to different sampling time, explant types and disinfection time. In addition, varieties of plants, organs and tissues contain different phenolic compounds in content and types, so explants of different plants appear brown in different degrees (Pindel and Miczynski, 1996). Among these factors, we suggested that hormone kinds and concentrations in the medium were crucial to stem tips germination, differentiation, growth, leaf callus induction and differentiation according to the results in this research. There were three reasons which could demonstrate this conclusion. Firstly, in this experiment, MS was used as the basic medium which was added with 6-BA and NAA for differentiation induction. When 6-BA concentration was 0.5 mg L^{-1}, the differentiation rate was almost 0, but when the concentration increased to 1.0 mg L^{-1}, the differentiation rate was up to 81.2%. Secondly, we used 6-BA or TDZ (whose concentration used was much lower than other cytokines) for multiplication induction (Huetteman et al., 2005; Wang et al., 2006). The results showed that, with the increasing concentration of TDZ in the mediums, both the proportion and the degree of vitrification of seedlings increased. When TDZ concentration was 0.01 mg L^{-1}, the seedlings grew well. At last, when we set six treatments for rooting induction, it was obvious that different basic medium types, especially different hormone kinds and concentrations had different effects on rooting induction of explants from *Malus* 'Indian magic' plants. All these stated indicated that, the rational use of hormones was an exceptionally important factor for the construction of plant regeneration system.

Establishment of efficient leaves *in vitro* regeneration system is important for genetic transformation of crabapple

In term of *Malus* tissue culture, adventitious buds induction directly from explants and from callus of explants such as stem tips and leaves are two important ways for regeneration. In the experiments of adventitious buds induction directly from leaves, it was found that both cytokinin and auxin were necessary for leaves regeneration induction; high concentration ratio of cytokinin to auxin resulted in high induction of leaves regeneration (Ou et al., 2008). According to recent studies (Szankowski et al., 2003; Holefors et al., 1998), it was found that adventitious buds induction directly from apple leaves held a considerable proportion. It was possible that bigger intercellular space helped differentiation of the adventitious buds from cells in Malus leaves. So Malus leaves were regarded as ideal acceptors for gene transfer operations and somatic mutation. Cheng et al. (1998) used leaves of *Malus domestica* as gene transfer receptor and successfully transferred *Gus* (beta-Glucuronidase) gene and *Bt* (*Bacillus thuringiensis* δ-endotoxin) target gene and ultimately got transgenic lines which contained target gene. Yu et al. (2008) used leaves explants from *in vitro* cultured *Malus xiaojineses* to establish the regeneration system for genetic transformation and polymerase chain reaction amplification (PCR). Southern blot analysis indicated that, target gene was integrated into *M. xiaojineses.* Therefore, the establishment of efficient leaves *in vitro* regeneration system becomes a key technical link for genetic transformation of Malus.

In this research, we used nine treatments to induce leaves callus of *Malus* 'Indian magic' plants and discovered that the optimum medium for leaves callus induction was MS containing 1.5 mg/l 6-BA plus 1 mg/l 2, 4-D, in which leaf callus induction rate was 100%. On the medium of MS containing 1 mg/l 6-BA and 0.1 mg/l NAA, leaves directly regenerated adventitious buds. These results may provide foundations for continuous improvement of tissue culture technology and construction of genetic transformation system of crabapple. Future research should focus on optimizing leaf regeneration system and genetic transformation system to improve transformation efficiency and obtain transgenic lines expected to cultivate new varieties of transgenic crabapple.

ACKNOWLEDGEMENTS

This work was funded by National Natural Science Foundation of China (31071785) and Funding Project for Academic Human Resources Development in Institutions of Higher Learning Under the Jurisdiction of Beijing Municipality (PHR20110515),and also funded by Youth fund of Beijing University of Agriculture. This work was supported by Tissue Culture Center and Fruit Tree Key Laboratory of Beijing University of Agriculture (BUA).We appreciate all technicians in Crabapple Germplas resource Center of BUA.

REFERENCES

Casanova E, Moysset L, Trillas MI (2008). Effects of agar concentration and vessel closure on the organogenesis and hyperhydricity of adventitious carnation shoots. Biologia Plantarum, 52 (1): 1-8.

Cheng JS, Tian YC, Meng XM (1998). Transformation of Bacillus thuringiensis δ-endotoxin gene into *Malus domestica*. Acta Botanica Boreali-Occidentalia Sinica, 8(1): 78-81.

De Klerk GJ, Keppel M, Ter Brugge J, Meekes H (1995). Timing of the phases in adventitious root formation in apple microcuttings.J Exp Bot., 46:965–972.

Han XJ, Yang HJ, Duan KX, Zhang XR, Zhao HZ, You SZ, Jiang QQ (2009). Sodium nitroprusside promotes multiplication and regeneration of *Malus hupehensis* in vitro plantlets. Plant Cell Tiss Organ Cult 96:29–34.

Holefors A, Zhong TX, Welander M (1998). Transformtion of the apple rootstock M26 with the *rolA* gene and its influence on growth. Plant Sci., 136:69-78.

Huetteman CA, Preece JE (2005). Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Organ. Cult., 33(2):105-119.

Kakani A, Li GS, Peng Z (2009). Role of AUX1 in the control of organ identity during in vitro organogenesis and in mediating tissue specific auxin and cytokinin interaction in *Arabidopsis*. Planta. 229(3): 645-657.

Kikkert JR, Hebert SD, Wallace PG, Striem MJ, Reisch BI (1996). Trangenic plantets of “Chancellor”grapevine (*Vitis* sp.) from biolistic transformation of embryogenic cell suspensions. Plant Cell Report, 15(5): 311-316.

Liu LL, Cai FH, Liu D, Li YL, Zhang Z (2007) .Establishment of a high efficient regeneration system *in vitro* from apple cultivar Starkrimson. J. Fruit Sci., 24(5): 679-681.

Ou QC, Li LG, He P, Zhang ZH (2008). In vitro adventitious shoot regeneration and induction of tetraploid from leaves of Hanfu apple[J]. J. Fruit Sci., 25(3):293-297.

Pindel A, Miczynski K (1996). Regeneration of Cymbidium orchidsfrom leaf and root explants. Floria Horticult., 8(2):95-105.

Szankowski I, Briviba K, Fleschhut J, Schönherr J, Jacobsen HJ, Kiesecker H (2003). Transformation of apple (*Malus domestica* Borkh.) with the stilbene synthase gene from grapevine (*Vitis vinifera* L.) and a *PGIP* gene from kiwi (*Actinidia deliciosa*).Plant Cell Rep., 22: 141–149.

Thorpe TA (1983). Morphogenesis and regeneration in tissue culture. Beltsville Sym. Agr. Res., 7: 285-303.

Wang Y, Zeng YL, He B, Li JY, Gao Y, Zhang FC (2006). Study on the Vitrification Mechanism of Rape Hypocotyl *in vitro*. Biotechnology, 16(1): 75-77.

Wei Q, Lu WD, Liao Y, Pan SL, Xu Y, Tang L, Chen F (2004). Plant regeneration from epicotyl explant of *Jatropha curcas*. J. Plant Physiol. Mol. Biol., 30(4): 475-478.

Yeh-Jin.A, Louisa V, Thomas AM, Grace QC (2007). High-frequency plant regeneration through adventitious shoot formation in castor (*Ricinus communis* L.).*In vitro* Cell. Dev. Biol. Plant, 43(1):9-15.

Yu YL, Wang Y, Han ZH, Li TZ, Kong J, Xu XF (2008). Agrobacterium rhizogen-mediated transformation of *Malus xiaojinensis* Cheng et Jiang. J. Nuclear Agric. Sci., 22(5):621-625.

Zhang HP, Zhang ZM, Fan XW, Zhang LX, Ma BG (2009). Stem apex tissue culture of anti-cold apple “new crown”. Northern Horticult., (2): 99-101.

Zhang QT, Xia Y, Song ZX, Liu YL, Chen XB (2007). Establishment of Regeneration System of *Malus halliana koehne* by Tissue Culture. Biotechnology, 17(3):73.

Zhang YP (1992). Callus Induction and Plant Regeneration From Leaves of *Malus Prunifolia*. Plant Physiol. Communt., (6): 435.

Zhou JH,Zhou JR,Zeng HS,Wang GB,Zhu ZP (2000). Browning phenomenon and anti-browning research in the tissue culture of horticultural plant. Acta Horticulturae Sinica, 27: 481-486.