STUDY ON *IN VITRO* PROPAGATION THROUGH MULTIPLE SHOOT PROLIFERATION IN WOOD APPLE (*Aegle marmelos* L.)

R. DAS, M. F. HASAN, M. S. RAHMAN, M. H. RASHID[1] AND M. RAHMAN

Department of Genetic Engineering and Biotechnology, [1]Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh

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ABSTRACT

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The experiment was carried out at Professor Ali Md. Eunus Laboratory, Department of genetic Engineering and Biotechnology, university of Rajshahi, Bangladesh, during the period of January to December 2007. An efficient protocol was devised for rapid *in vitro* propagation through multiple shoot induction from cotyledons and shoot tips of wood apple (*Aegle marmelos*). The highest percentage of multiple shoots was 91.23% obtained in MS medium augmented with 2.0 mgl^{-1} BAP+0.2 mgl^{-1} NAA and the maximum number of multiple shoots was 22.7 per culture obtained in MS medium enriched with 2.0 mgl^{-1} BAP+0.2 mgl^{-1} NAA within fourteen days of inoculation. The induced shoots were elongated on the same medium. The elongated shoots were separated and cultured for root induction. Rooting of *in vitro* raised shoots was best induced on half strength MS medium supplemented with 1.0 mgl^{-1} IBA with highest percentage of shoot regenerating roots (80.42%) with 4 roots per shoot. The well rooted plantlets were acclimatised and successfully established onto the natural condition with 80% survival.

Key words: Multiple shoot, cotyledons, shoot tips, wood apple

INTRODUCTION

Wood apple (*Aegle marmelos*) is one of the most important medicinal plant as well as a fruit plant throughout the tropical countries. This plant is a member of the family Rutaceae. In Bangladesh it is known as Bael (Huq, 1986). It is an old native of Indian tropical region and now cultivated throughout Southeast Asia and East Indian Archipelago (Purseglove, 1968). It is a deciduous tree having profuse dimorphic branch; alternate, trifoliate, deep green leaves; membranous leaflets; large, sweet scented, greenish white flowers; and large, globose or ovoid or pyriform fruit (Prain, 1963). The ripe fresh fruits are eaten and it's juice is used as soft drinks, for making candy, squash, pulp powder and nectar (Misra, 1999) unripe fruits are used for making marmelle oil (Samad, 1966) and baelshut (Hassan, 1988) which may be valuable for medicine. The edible portion of the flesh contain water, protein, starch, fat, mineral salt, carotene, niacin, vitamin B_1, vitamin B_2, vitamin C, calcium and iron (Khan and Haque, 1975). Different parts of the tree also contains certain biochemical constituents namely alkaloids, aegelinol, coumarin, steroid (Misra, 1999), terpenoid (Rana *et al.* 1997) and tannin (Parichha, 2004). The plant has been widely used for its having antibacterial (Chattopadhyay, 2008), antifungal (Rana *et al.* 1997), antioxidant (Dhalwal *et al.* 2008), antidiarrhoetic (Mazumder *et al.* 2006), pesticidal, antidote, anti-inflammatory properties (Misra, 1999). Fresh half ripe wood apple is mildly astringent and used to cure dysentery, diarrhoea, hepatitis, tuberculosis, dyspepsia and also beneficial for heart and brain (Misra, 1999). Various parts of the plants are also used for treating anaemia, wound healing, high blood pressure, asthma, jaundice, and troubles during pregnancy, typhoid (Paricha, 2004) and diabetes (Narendhirakannan, 2005). It has also timber value and provides good quality firewood.

Wood apple is propagated either through seeds or vegetatively by root cutting or sucker formation, which is a rare occurrence (Singh and Roy, 1984). This plant usually infested by several insects such as, *Phyllocnistis citrella, Aonidiella aurantii* and *Papilio demoleus* and also some disease namely bacterial shoot hole, fruit canker and gummosis which caused a great loss in its yield (Morton, 1987). Since wood apple is a cross pollinated plant, maintenance of varietal purity is one of the important problems. Due to the presence of enormous heterozygosity in the most of the existing varieties, sibling pollination may produce inbreeding depression. Micropropagation of wood apple may play an important role in solving this problem through rapid *in vitro* multiplication of particular genotype. In recent years there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare endangered and threatened medicinal plants (Anis and Faisal, 2005). Further genetic improvement is another approach to augment the drug yielding capacity of the plant (Mulabagal and Tsay, 2004). Therefore, it is important to conserve wood apple and protect it extinction. There are many reports on media composition and potential technology for mass scale production of several medicinal and fruit plants, such as *Azadirachta indica* (Kabir *et al*. 1994), *Adhatoda vasica* (Banu *et al.* 1997), *Centella asiatica* (Nath and Buragohain, 2003; Mohapatra *et al*. 2008), *Aristolochia indica* (Manjula *et al*. 1997) and *Carica papaya* (Islam *et al*. 2000). So far our knowledge goes; there is no suitable report on *in vitro* propagation of wood apple. The

objective of this present study was to establish a reproducible protocol for mass micropropagation and preservation of this valuable medicinal plant resource.

MATERIALS AND METHODS

For this experiment, cotyledon from mature seeds and shoot tip from *in vitro* grown plantlets were used. Seeds from mature fruits of wood apple were collected from Rajshahi University campus, Bangladesh. The seeds were washed thoroughly in running tap water for 30 minutes and then soaked in Tween-80 and Savlon for 10 minutes. Then the seeds were washed in distilled water for several times. Finally, the seeds were rinsed in 0.1% $MgCl_2$ for 10 minute and washed with autoclaved distilled water for several times to remove the traces of sterilant. The sterilized seeds were then excised to remove the cotyledons. Each of the cotyledons was excised into 2 pieces and each piece was then cultured separately in different culture tubes having MS (Murashige and Skoog, 1962) medium enriched with different concentrations and combinations of BAP and NAA. For another experiment, the sterilized seeds were inoculated to sterile seed germinating medium (MS) in culture bottle. Shoot tips were excised from two week old *in vitro* grown seedlings and inoculated onto MS medium fortified with different concentrations and combinations of BAP and NAA.

Throughout the experiments, both full strength MS medium and half strength MS medium with 3% (W/V) sucrose and gelled with 0.8% (W/V) agar was used. In all cases, the pH of the medium was adjusted to 5.7 before autoclaving and adding agar. About 10 ml of the medium were dispensed in each culture tube and sealed with non-absorbent cotton plugs prior to autoclaving at 121°C for 21 minutes. The cultures were incubated in a culture room at 25 ± 2^0C with a photoperiod of 16 hour at 3000 lux light intensity provided by cool white fluorescent tubes. Explants were cultured on full strength of MS medium with different concentrations and combinations of BAP and NAA for multiple shoot induction. Root induction from the developing shoots was achieved on half strength MS medium supplemented with different concentrations of IBA. After 35 days, well rooted plantlets were obtained. Subsequently, the plantlets were removed from the culture vessels, washed gently under running tap water and planted in pots containing sterile sand, soil and humus at the ratio of 1:2:2. The potted plantlets were covered by polythene sheet to maintain suitable humidity. After sufficient acclimatization, the plantlets were transplanted in the field condition, where 80% plants were survived.

RESULTS AND DISCUSSION

Proliferation of multiple shoots was observed with high frequency from cotyledons and shoot tips within fourteen days of inoculation. These explants were capable of directly developing multiple shoot on MS medium containing different concentrations and combinations of auxin and cytokinin.

The highest percentage of multiple shoot induction was 91.23% on the medium augmented with 2.0 mgl^{-1} BAP+0.2 mgl^{-1} NAA (Figure 1A-B, Table 1) followed by 78.59% on the medium consisting of 2.0 mgl^{-1} BAP+0.1 mgl^{-1} NAA from cotyledon. On the other hand, the lowest percentage of multiple shoots induction was found to be 14.29% from cotyledon on the media supplemented with 0.5 mgl^{-1} BAP+0.1 mgl^{-1} NAA. The maximum number of shoots was 22.7 per explants obtained on the medium having 2.0 mgl^{-1} BAP+0.2 mgl^{-1} NAA (Table 1) followed by 17.2 shoots per explants in the medium containing 2.0 mgl^{-1} BAP+0.1 mgl^{-1} NAA from cotyledon. On the contrary, the minimum number of shoots was 2.0 per explants on the medium consisting of 0.5 mgl^{-1} BAP + 0.3 mgl^{-1} NAA from cotyledon.

In case of shoot tips explants, the highest percentage of multiple shoot induction was 86.36% on the medium augmented with 2.0 mgl^{-1} BAP+0.3 mgl^{-1} NAA (Table 1, Figure 1C-D) followed by 82.49% on the medium consisting of 2.0 mgl^{-1} BAP+0.2 mgl^{-1} NAA. On the left hand, the lowest percentage of multiple shoot induction was found to be 14.50% from shoot tips on the media supplemented with 0.5 mgl^{-1} BAP+0.2 mgl^{-1} NAA. The highest number of shoots was 20.5 per explants obtained on the medium having 2.0 mgl^{-1} BAP+0.3 mgl^{-1} NAA followed by 16.6 per explants in the medium fortified with 2.0 mgl^{-1} BAP+0.5 mgl^{-1} NAA from shoot tips. On the contrary, the lowest number of shoots was 2.0 per explants on the medium supplemented with 0.5 mgl^{-1} BAP + 0.2 mgl^{-1} NAA from shoot tips. The induced shoots were elongated in the same medium. In the present investigation, MS medium augmented with 2.0 mgl^{-1} BAP+0.2 mgl^{-1} NAA found to be the best treatment for maximum multiple shoot induction as well as maximum number of shoots per explants. Cotyledon was found to be superior for maximum multiple shoot induction as well as maximum number of shoots per explants. Similar results were also reported in several medicinal and fruit plants, such as *Azadirachta indica* (Kabir *et al*. 1994), *Adhatoda vasica* (Banu *et al*. 1997), *Centella asiatica* (Nath and Buragohain, 2003; Mohapatra *et al*. 2008), *Aristolochia indica* (Manjula *et al*. 1997).

Well developed shoots were isolated and cultured onto MS media having different concentrations of IBA for root induction. The highest parentage of root induction was 80.42% on the half strength of MS medium augmented with 1.0 mgl^{-1} IBA (Figure 1E, Table 2) followed by 63.64% on the medium with 0.5 mgl^{-1} IBA. On the other hand, the lowest percentage was 10.45% on the medium supplemented with 2.5 mgl^{-1} IBA. The highest number of roots per shoots was 4.0 from the medium consisting of 1.0 mgl^{-1} IBA. On the contrary, the lowest number of roots per shoot was 1.0 in the medium fortified with 0.1 mgl^{-1} IBA. Thus, 1.0 mgl^{-1} IBA was found to be an ideal treatment for root induction. Similar results were also reported in several medicinal and fruit plants, such as *Carica papaya* (Islam *et al*. 2000), *Centella asiatica*(Mohapatra *et al*. 2008), *Ocimum basilicum*(Sahoo *et al.* 1997), *Gymnema sylvestre* (Komalavalli and Rao, 2000) *Holostemma adakodien* Schult (Martin, 2002) *Swainsona salsula*(Yang *et al*. 2001). After 35 days, well rooted plantlets were obtained. Subsequently, the plantlets were removed from the culture vessels, washed gently under running tap water and planted in pots containing sterile sand, soil and humus in the ratio of 1:2:2 (Figure 1F). The potted plantlets were covered by transparent polythene sheet to maintain suitable humidity. After sufficient acclimatization, the plantlets were transplanted in the natural condition, where 80% plants were survived. In the present experiment, a fruitful protocol was set up through multiple shoot induction from cotyledon and shoot tip. This protocol can be exploited for commercial propagation and conservation of valuable medicinal and fruit plant resources.

Table 1. Effect of different concentrations and combinations of BAP and NAA on multiple shoot induction from cotyledons and shoot tips of wood apple (*Aegle marmelos*)

Hormone concentrations (mg/l)		Source of explants					
		Cotyledons			Shoot tips		
BAP+NAA		Multiple shoot induction (%)	No. of shoots/ explants (M±SE)	Shoot length (cm) (M±SE)	Multiple shoot induction (%)	No. of shoots/ explants (M±SE)	Shoot length (cm) (M±SE)
0.5	0.1	14.29	3.7±0.7	3.6±0.6	18.18	5.7±0.7	4.3±0.3
	0.2	35.84	4.2±0.2	4.3±0.3	14.50	2.0±0.5	5.5±0.5
	0.3	18.00	2.0±0.1	5.5±0.5	45.52	9.5±0.5	5.6±0.6
	0.5	28.57	3.1±0.6	4.4±0.4	36.87	6.2±0.2	4.4±0.5
1.0	0.1	50.00	7.5±0.5	5.1±0.1	30.59	13.0 ±0.5	5.7±0.7
	0.2	71.45	10.2±0.3	5.7±0.7	56.34	14.5±0.5	5.5±0.5
	0.3	40.00	7.5±0.5	5.8±0.8	72.73	15.5±0.5	6.1±1.0
	0.5	21.12	8.3±0.3	4.3±0.4	63.56	11.0±1.0	5.3±0.3
1.5	0.1	35.64	6.7±0.7	5.1±0.1	18.27	5.5±0.5	5.8±0.8
	0.2	57.25	8.6±0.6	4.9±0.9	32.14	9.7±0.7	3.7±0.5
	0.3	75.53	4.1±0.8	4.3±0.3	65.32	14.4±0.4	4.3±0.7
	0.5	43.57	6.9±0.9	3.7±0.7	24.28	12.6±0.5	3.6±0.4
2.0	0.1	78.59	17.2±1.0	5.8±0.8	81.85	14.7±0.5	5.4±0.6
	0.2	91.23*	22.7±0.5*	6.2±0.2	82.49	15.9±0.9	5. 7±0.7
	0.3	60.28	11.5±0.5	5.7±0.7	86.36	20.5±0. 5	6.1±0.5
	0.5	64.34	9.5±0.5	3.6±0.6	68.19	16.6±7	5.6±0.4
2.5	0.1	35.78	7.6±0.6	3.7±0.7	63.64	14.1±2	5.7±0.7
	0.2	51.00	6.3±0.3	5.2±0.2	69.26	12.1±8	5.2±0.5
	0.3	30.35	5.7±0.7	5.8±0.8	32.45	9.3±5	5.3±0.7
	0.5	32.01	6.1±0.3	4.9±0.9	21.03	5.9±6	4.8±0.8

Each value represents an average of 10 replicates and each experiment was repeated at least thrice

Table 2: Effect of IBA in half-strength MS medium on root induction in regenerated shoots

IBA (mg/l)	Root induction (%)	Number of roots/shoot (M±SE)	Root length (cm) (M±SE)
0.1	40.37	1.8±0.8	1.6±0.3
0.5	63.64	2.9±0.9	2.7±0.9
1.0	80.42	4.0 ±1.0	3.5±0.5
1.5	30.24	3.7±0.7	2.4±0.4
2.0	20.35	2.5±0.5	1.3±0.6
2.5	10.45	1.0±0.8	0.8±0.7

Each value represents an average of 10 replicates and each experiment was repeated at least thrice.

Figure 1. Rapid proliferation of multiple shoots from cotyledon and shoot tip.
A. Multiple shoots initiation from cotyledons.
B. Multiple shoots elongation from cotyledons
C. Multiple shoots initiation from shoot tips
D. Multiple shoots elongation from shoot tips
E. Induction of roots on regenerated shoots
F. An acclimatized plant under natural condition

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