International Journal of Farm Sciences **13(1):** 98-107, 2023; **doi: 10.5958/2250-0499.2023.00020.4**
Winner of SADHNA Best Paper Award 2023

Optimization of critical determinants for efficient transformation in M7 apple rootstock

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 Received: 23.02.2023/Accepted: 25.03.2023

ABSTRACT

Optimizing the critical determinants for achieving efficient genetic transformation in apple has been considered as the first and foremost step. Therefore, in the current study, effect of different cytokinins-auxins, antibiotics, pre-culturing, co-cultivation duration and infection time were investigated in an effort to increase the efficiency of producing putative transformants in M7 apple rootstock. The findings demonstrated that MS medium supplemented with 4 mg/l BA and 0.5 mg/l NAA resulted in 20 per cent shoot regeneration, whereas, 0.6 mg/l TDZ with 0.5 mg/l NAA in 41.46 per cent regeneration. Cefotaxime at low concentration (200-300 mg/l) was not only non-toxic but also stimulated shoot regeneration. *Agrobacterium tumefaciens* strain LBA4404 containing pCAMBIA 2300 plasmid encoding neomycin phosphotransferase II and *rolB* gene were used for leaf transformation. It was found that pre-culturing for 48 hours, an infection time of 7 min and co-cultivation for 48 hours resulted in induction of callus/ shoots. To overcome bacterial overgrowth after co-cultivation, 500 mg/l cefotaxime was found appropriate.

Keywords: Apple; BA; TDZ; transformation protocol; antibiotic sensitivity; *Agrobacterium*

INTRODUCTION

Since last few decades, most apple cultivation is done on clonal rootstocks as they are useful for retaining unique attributes and effects on scion varieties such as growth, disease resistance and uniformity. Malling 7 is a semi-dwarf rootstock with good commercial value in Indian condition, adaptable to wide range of soil types and temperatures and withstand temporary drought. This rootstock is susceptible to soil-borne diseases like white root rot and collar rot and less susceptible to replant disease. Pathogens, such as bacteria, fungi, nematodes and actinomycetes, which deprive newly planted trees of their essential nutrients and cause maladies on their root systems, are responsible for the soil-borne disease syndrome known as Replant Disease in apples. Their growth is negatively impacted, as a result, rendering plantations uneconomical.

There are some reports showing that *rolB* gene found in T-DNA of Ri plasmid of *Agrobacterium rhizogenes* improves the rooting ability of woody plants *(*Zhu et al 2001) by increasing the auxin sensitivity of the tissue (Shoja 2010). It has successfully been transformed in apple rootstock M26 (Welander et al 1998), Jork 9 (Sedira et al 2001), M9 (Zhu et al 2001) and apple scion cultivar Florina (Radchuck and Kurkhovoy 2005) to increase the rooting potential. Transgenic plants resulted in increased number of roots per shoot which absorbed more nutrients from soil ultimately increased yield and productivity.

For genetic transformation in apples, a highly efficient plant regeneration protocol is required. Apple genotypes with a low regeneration capacity are extremely difficult to transform.

However, a number of researchers have reported high regeneration capacity and genotype selection to set up efficient shoot regeneration procedures using leaf explants in a variety of apple cultivars and rootstocks (James et al 1988, Magyar-Tabori et al 2010, Arcos et al 2020).

The impact of antibiotics on sensitivity of plant tissue must be determined before performing the transformation trials because the sensitivity of plant tissue is dependent on a number of factors viz species, genotype, type of explant and culture conditions (Farzaneh et al 2013). Excessive dose of antibiotics hinders the regeneration and proliferation of transformed tissues, but inadequate dose leads to a high rate of untransformed escapes and chimeras. There concentrations for apple leaf explants are highly genotypic. Antibiotics from β-lactam family, like cefotaxime, have negligible harmful effects on plant tissues (Chevreau et al 1997). It is highly resistant to β-lactamases and inhibits bacterial cell wall synthesis (Wang et al 2010) and most commonly used antibiotic to eradicate *Agrobacterium* from cultures.

Aminoglycosides like kanamycin are the most frequently used selectable markers during transformation. It is inactivated by phosphorylation by the homologous resistance gene *nptII*, which encodes an aminoglycoside 3'-phosphotransferase (Miki and McHugh 2004). It is not only harmful but can also affect a plant regeneration response (Mahadev et al 2014). In order to obtain a large number of positive transgenic lines, consistent and genotype-independent approaches are needed. Furthermore, *Agrobacterium* must also be successfully eliminated from the cultures as soon as it is no longer needed using the appropriate antibiotic.

The purpose of this work was to achieve the improved regeneration protocol and to optimize the minimal antibiotic dose to be added in culture media prior to successful transformation trials with *rolB* gene. Keeping it in view, the effect of antibiotic concentration on regeneration ability and to restrict the *Agrobacterium* growth was optimized that may facilitate the selection of transformed shoots.

MATERIAL and METHODS

Plant material and *Agrobacterium* strain: Four to five apical young leaves excised from 4 weeks old in vitro multiple shoots of apple rootstock M7 were used as explants for regeneration and co-cultivation. *A tumefaciens* strain LBA4404 harboring pCAMBIA 2300 vector with *nptII* as a selectable marker and *rolB* as target gene, both under the control of 35S cauliflower mosaic virus (CaMV) promoter, were used for co-cultivation experiments.

Shoot induction from leaf explants: Leaf explants were cultured on shoot regeneration medium supplemented with various combinations of auxins (0.5-1 mg/l NAA/IAA/IBA) and cytokinins (3-5 mg/l BA/ 0.4-1.0 mg/l TDZ). After six weeks, the frequency of regeneration and number of shoots per regenerating explant were recorded.

Micropropagation of regenerants: For multiplication, regenerated shoots from the leaves/callus were separated and cultured on shoot multiplication medium ie MS medium supplemented with 0.5 mg/l BA, 0.5 mg/l GA_3 and 0.1 mg/l IBA. Regenerated shoots of 1-2 cm length were used for rooting on 1/2 st MS medium with 0.1-0.5 mg/l IBA. For acclimatization, rooted plantlets were transplanted into protrays filled with autoclaved soil:cocopeat (1:1). Humidity and temperature were maintained in the polyhouse.

Antibiotics sensitivity experiments: Control and transformed leaves were tested for regeneration response on antibiotic supplemented medium. Different concentrations of kanamycin 20-40 mg/l alone and in combination with cefotaxime 200-500 mg/l were added onto the regeneration medium (MS+4 mg/l BA+ 0.5 mg/l NAA or 0.6 mg/l TDZ + 0.5 mg/l NAA). Leaf segments, that turned brown or formed calli and/or adventitious shoots, were recorded.

Optimizing pre-culturing, infection and co-cultivation duration: Leaf segments were pre-conditioned for 0, 24 or 48 h on regeneration medium containing 0.6 mg/l TDZ and 0.5 mg/l NAA under light before being infected and incubated with *A tumefaciens* to identify the better duration of pre-culture. With gentle pricking and stirring, pre-cultured leaf segments were infected with *Agrobacterium* cell suspension for 5-8 minutes to determine the influence of infection time on the intensity of bacterial growth. The leaf segments were placed on basal MS media after eliminating the excess bacteria by blotting dry on sterile filter paper. They were co-cultivated in dark for 48, 72 and 96 h.

Following co-cultivation, explants with bacterial growth at their margins were rinsed with half strength MS basal medium containing 250 mg/l cefotaxime, blotted dry on sterile filter paper, transferred to fresh regeneration medium supplemented with 500 mg/l cefotaxime and 35 mg/l kanamycin and incubated in light. Every alternate day, the growth of *Agrobacterium*

was monitored. Rinsing and blotting were repeated of explants again developed bacterial growth. After 15 days, the concentration of cefotaxime was reduced from 500 to 400 mg/l, then gradually to 200 mg/l, to allow transformed tissue to regenerate adventitious shoots. On both selective and non-selected media, the performance of putative transformed cells or shoots was compared to that of control. Observations were made based on morphological evidence, such as *Agrobacterium* growth and average number of calli or shoots produced from the explants that survived.

RESULTS

Effect of BA with auxins: Different BA concentrations were combined with variable concentrations of three auxins (NAA/IAA/IBA) to evaluate their physiological effect on shoot regeneration frequency. It is inferred that highest shoot regeneration ie 20.00 ± 0.58 was observed on MS medium supplemented with 4 mg/l BA and 0.5 mg/l NAA (Table 1, Fig 1A), while media supplemented with 5 mg/l BA and 1 mg/l IAA had the lowest regeneration efficiency of 5.88 ± 0.35. There was no significant difference between the combination of 5 mg/l BA with 0.5 mg/l NAA, which resulted in 15.00 ± 0.58 shoot regeneration frequency and 4 mg/l BA with 1 mg/l IAA, which induced 13.16 ± 0.58 shoot regeneration frequency. In BA and NAA treatments, the percentage of explants that produced callus ranged from 37.5 to 92.86 per cent. The calli ranged in morphology from compact, yellowish green to light greenish. BA with IAA or IBA combinations also resulted in calli formation with rates ranging from 8.33 to 83.33 per cent. Therefore, it is inferred that high concentration of BA with low NAA can lead to higher shoot regeneration.

Effect of TDZ with NAA/IAA/IBA: Among all the evaluated treatments of TDZ with NAA/IAA/IBA, highest shoot regeneration ie 41.46 ± 0.59 (direct and indirect) was achieved on medium with 0.6 mg/l TDZ and 0.5 mg/l NAA (Table 1, Fig 1B). The percentage of explants with greenish white, compact and granular calli ranged from 53.85-100 per cent. Addition of 0.5 mg/l IAA and 1 mg/l NAA each with 0.8 mg/l TDZ resulted in similar regeneration efficiency of 36.36 per cent.

Therefore, it is inferred from the present study that higher dosage of TDZ was found to reduce the regeneration percentage. In some of the TDZ combinations, vitrified and abnormal shoots originated from callus with more dissected leaves, while others were healthy, green and originated directly. Regeneration percentage was affected by NAA and IAA levels, however, the number of shoots per explant was minimal.

Regenerated shoots multiplied 5-6 times per inoculated shoot (Fig 1C). These shoots were successfully hardened after being rooted on 0.2 mg/l IBA (Fig 1D). The rooting rate of regenerants was roughly 25-50 per cent, whereas, hardening success of 100 per cent was observed (Fig 1E).

Effect of antibiotic sensitivity: Leaf explants cultured on regeneration medium (MS with 0.6 mg/lTDZ and 0.5 mg/l NAA) supplemented with 20-40 mg/l kanamycin started turning brown after 3-4 days. At 20 mg/l, 4.17 ± 0.07 average explants turned pale yellow, increasing to 95.83 ± 0.51 at 40 mg/l (Table 2, Fig 2), indicating that with increase in kanamycin dose, explants survival rate decreased progressively. However, callus induction was observed only at 20 mg/l kanamycin, but at higher doses, it was decreased. No shoot was induced in any of the combinations while 100 per cent callus induction and 39.39 ± 0.65 average shoot regeneration was achieved on control medium. A lethal dose of 35 mg/l kanamycin was chosen for selection of transformed tissue.

Callus induction decreased with the increase in cefotaxime from 200 to 500 mg/l (Table 2, Fig 2). Callus was white and compact at first and then turned green and later brown at certain areas. At 200 and 300 mg/l, 33.33 ± 0.55 and 8.33 ± 0.11 per cent shoot regeneration was obtained from callus respectively. Whereas, at higher doses (400 and 500 mg/l), callus formation was promoted and shoot regeneration was suppressed. To observe the combined effect of kanamycin and cefotaxime, a few leaf explants were cultured on medium with 20-25 mg/l kanamycin and 200-300 mg/l cefotaxime (Table 3). It was observed that after three weeks of culturing, certain parts of explants gradually turned pale/brown (Fig 2). No callus or shoot regeneration was induced in any of these combinations.

After co-cultivation, *Agrobacterium* overgrowth could not be controlled effectively with lower doses of cefotaxime ie 200 and 300 mg/l added in the medium. As a result, leaf explants died. Whereas, 400 mg/l cefotaxime was found effective to suppress it to some extent and resulted in callus

Table 1. Effect of different combinations of plant growth regulators on callus/shoot regeneration from leaf explants of M7 apple rootstock

Plant growth regulator (mg/l)					Average callus induction (%)	Averages shoot regeneration (%)	Average number of shoots/explant
BA	TDZ	NAA	IAA	IBA			
3.0	-	0.5	-	-	37.50 ± 0.58^{q}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	0.5	-	-	92.8 ± 0.44^{c}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
3.0	-	1.0	-	-	47.83 ± 0.50^{n}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	1.0	-	-	66.67 ± 0.59^{l}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
3.0	-	-	-	0.5	71.43 ± 0.57^{j}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	0.5	-	-	80.00 ± 0.57^{g}	20.00 ± 0.58^{d}	1.50 ± 0.05^{cd}
5.0	-	0.5	-	-	90.00 ± 0.52^{d}	15.00 ± 0.58^{e}	1.50 ± 0.05^{cd}
3.0	-	-	0.5	-	83.33 ± 0.66^{f}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	-	0.5	-	39.22 ± 0.46^{p}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
5.0	-	-	1.0	-	8.82 ± 0.65^{r}	5.88 ± 0.35^{g}	1.00 ± 0.01^{e}
3.0	-	-	1.0	-	0.00 ± 0.00^{s}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	-	1.0	-	76.32 ± 0.73^{i}	13.16 ± 0.58^{f}	1.67 ± 0.12^{e}
3.0	-	-	-	1.0	78.57 ± 0.65^{gh}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	-	-	0.5	70.59 ± 0.61^{j}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	-	-	1.0	75.00 ± 0.58^{i}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.0	-	-	1.5	-	8.33 ± 0.57^{r}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.5	-	-	1.0	-	40.90 ± 0.60^{o}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
4.5	-	-	1.5	-	68.75 ± 0.59^{k}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
-	0.4	-	-	0.5	54.41 ± 0.47^{m}	5.88 ± 0.58^{g}	1.33 ± 0.16^{d}
-	0.6	-	0.5	-	94.59 ± 0.53^{b}	21.62 ± 0.60^{c}	1.60 ± 0.06^{c}
-	0.8	-	0.5	-	100.00 ± 0.00^{a}	36.36 ± 0.64^{b}	2.00 ± 0.00^{b}
-	1.0	-	0.5	-	92.85 ± 0.53^{c}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
-	0.6	-	1.0	-	72.22 ± 0.62^{j}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
-	0.8	-	1.0	-	53.85 ± 0.53^{m}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
-	1.0	0.5	-	-	85.71 ± 0.52^{e}	0.00 ± 0.00^{h}	0.00 ± 0.00^{f}
-	0.6	0.5	-	-	78.05 ± 0.57^{h}	41.46 ± 0.59^{a}	2.27 ± 0.17^{a}
-	0.8	1.0	-	-	81.81 ± 0.56^{f}	36.36 ± 0.49^{b}	2.22 ± 0.13^{a}
$CD_{0.05}$					1.56	0.92	0.17

induction in a few explants. However, it required washing and blotting of explants every other day which was time consuming. It was further observed that 500 mg/l cefotaxime successfully inhibited bacterial overgrowth (Fig 2) and gradual reduction from 400 to 200 mg/l resulted in 3.57 ± 0.07 per cent shoot regeneration.

Effect of pre-culturing, infection and co-cultivation duration: In present experiments, pre-culturing of leaf explants before transformation was found an important component that influenced T-DNA delivery. Increasing co-cultivation time from 2 to 3 days markedly increased the *Agrobacterium* overgrowth, which required frequent washing, blotting and sub-culturing. It led to decrease in average per cent callus induction. In was observed that fresh leaves (without pre-culturing) co-cultivated for 48, 72 and 96 h resulted in 68.66 ± 0.59, 66.00 ± 0.48 and 60.00 ± 0.47 per cent callus induction respectively (Table 4). Whereas, leaf explants pre-cultured for 24 h and co-cultivated for 48, 72 and 96 h resulted in 80.00 ± 0.09, 77.5 ± 0.53 and 72.5 ± 0.53 per cent callus induction respectively. Likewise, leaf explants pre-cultured for 48 h and co-cultivated for 48, 72 and 96 h induced 95.00 ± 0.47, 87.50 ± 0.76 and 80.00 ± 0.09 per cent callus respectively. Therefore, on the basis of observations taken from above experiments, it is inferred that leaf explants pre-cultured for 48 h and co-cultivated for 48 h resulted in maximum callus induction with 3.57 ± 0.07 per cent shoot regeneration (Table 4).

The optimum *Agrobacterium* infection has been proven to enhance the *Agrobacterium* attachment to the targeted explants. Infection for 5 and 6 minutes exhibited negligible or little agrobacterial growth on the edges of most leaf explants, whereas, 8 minutes promoted uncontrolled or vigorous bacterial growth which resulted in browning of explants. It was observed that the infection time of 7 min was found

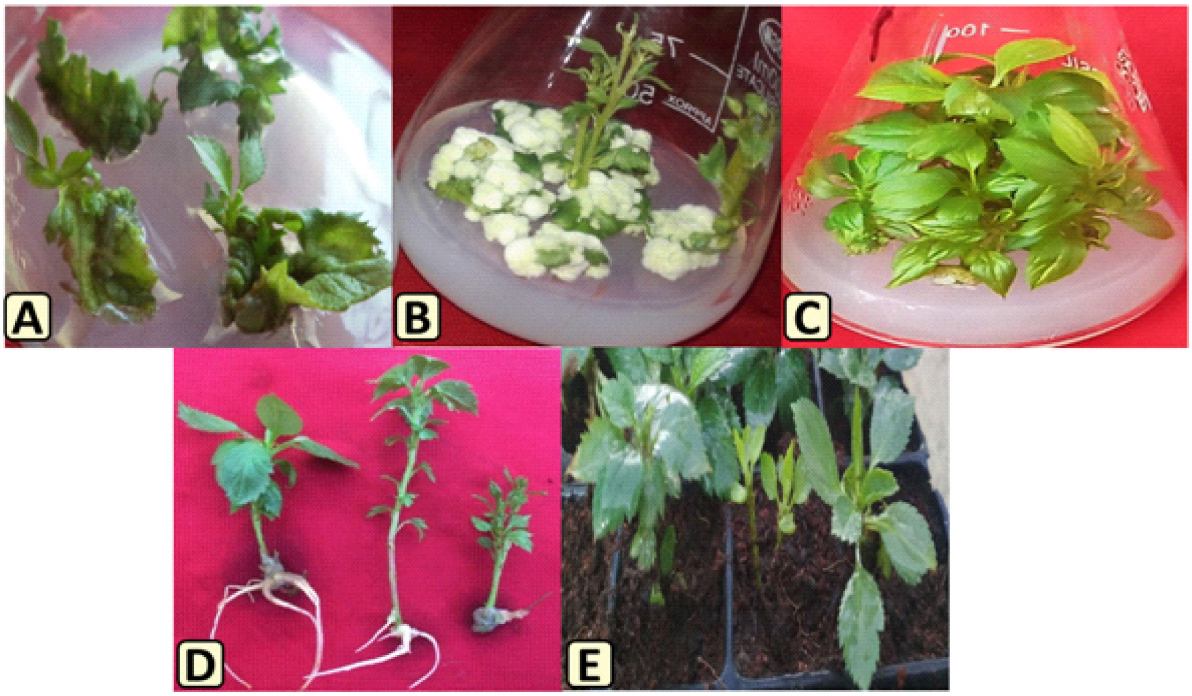

Fig 1. (A) Direct shoot regeneration on MS + 4 mg/l BA + 0.5 mg/l NAA, (B) Indirect shoot regeneration on MS + 0.6 mg/l TDZ + 0.5 mg/l NAA, (C) Multiplication of regenerants, (D) Rooting of microshoots, (E) Hardening of plantlets

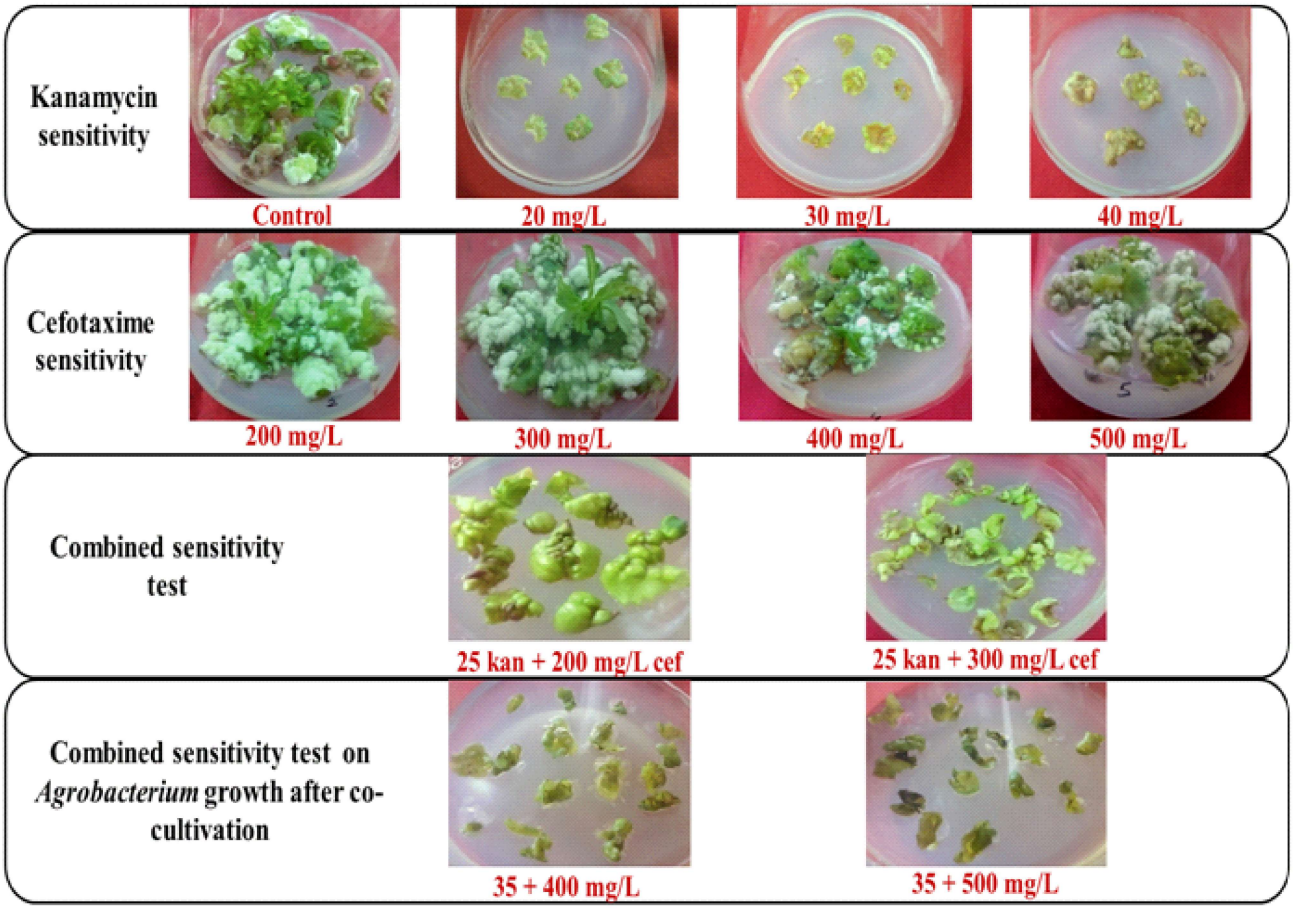

Fig 2. Antibiotic sensitivity test on leaf explants before and after co-cultivation with *Agrobacterium*

Table 2. Effect of different concentrations of kanamycin and cefotaxime on callus/shoot induction

Medium used: MS + 0.6 mg/l TDZ + 0.5 mg/l NAA Control		Average explants died (%) -		Average callus induction (%) 100.00		Average shoot regeneration (%) 39.39 ± 0.65[a]		Average number of shoots/explant 2.12 ± 0.00[a]
Kanamycin (mg/l)	Cefotaxime (mg/l)	Kanamycin	Cefotaxime	Kanamycin	Cefotaxime	Kanamycin	Cefotaxime	Cefotaxime
20	200	4.17 ± 0.07[e]	-	90.00+	100++	-	33.33 ± 0.55[b]	2.00 ± 0.01[b]
25	300	12.50 ± 0.69[d]	**	Only swelling	++	-	8.33 ± 0.11[c]	1.00 ± 0.07[c]
30	400	45.83 ± 0.60[c]	***	Only swelling	++	-	0.00 ± 0.00[d]	0.00 ± 0.00[d]
35	500	79.17 ± 0.52[b]	****	No swelling	++	-	0.00 ± 0.00[d]	0.00 ± 0.00[d]
40		95.83 ± 0.51[a]		No swelling		-		
$CD_{0.05}$		0.52					1.23	0.09

Explants turned brown: ** = Some part, *** = Half part, **** = Maximum part, + = Little callus, ++ = Callus on green areas

Table 3. Combined effect of kanamycin and cefotaxime on callus/shoot induction from leaf explants with or without co-cultivation

Without co-cultivation				After co-cultivation				
Medium used: MS + 4 mg/l BA + 0.5 mg/l NAA		Per cent explants turned brown	Average % of callus/shoot induction	Medium used: MS+0.6 mg/l TDZ+ 0.5 mg/l NAA		Growth of bacteria	Average % shoot regeneration	Average number of shoots/explant
Kanamycin (mg/l)	Cefotaxime (mg/l)			Kanamycin (mg/l)	Cefotaxime (mg/l)			
0	0	0.00 ± 0.00[e]	19.57 ± 0.52[a]	0	0	+++	0.00 ± 0.00[b]	0.00 ± 0.00[b]
20	200	25.00 ± 0.58[d]	0.00 ± 0.00[b]	35	200	++	0.00 ± 0.00[b]*	0.00 ± 0.00[b]
20	300	30.00 ± 0.27[c]	0.00 ± 0.00[b]	35	300	++	0.00 ± 0.00[b]	0.00 ± 0.00[b]
25	200	37.50 ± 0.62[b]	0.00 ± 0.00[b]	35	400	++	0.00 ± 0.00[b]	0.00 ± 0.00[b]
25	300	42.50 ± 0.31[a]	0.00 ± 0.00[b]	35	500	+	3.57 ± 0.07[b]	1.00 ± 0.30[a]
$CD_{0.05}$		1.34	0.74				0.09	0.43

+ = Growth only on leaf margins, ++ = Less growth, +++ = Excess growth

Table 4. Effect of pre-culturing, co-cultivation duration and infection time on organogenesis from leaf explants on MS medium with 0.6 mg/l TDZ + 0.5 mg/l NAA

Pre-culturing duration (h)	Co-cultivation time (h)	Per cent explants turned brown	Average per cent callus induction	Average per cent shoot regeneration	Time of dip in bacterial suspension (min)	Degree of infection	Average per cent shoot regeneration	Average number of shoots/explant
0	48	35.00 ± 0.55^{a}	68.66 ± 0.59^{f}	0.00 ± 0.00^{b}	0	-	37.95 ± 0.57^{a}	1.67 ± 0.05^{a}
	72	27.50 ± 0.38^{b}	66.00 ± 0.48^{g}	0.00 ± 0.00^{b}				
	96	25.00 ± 0.61^{c}	60.00 ± 0.47^{h}	0.00 ± 0.00^{b}	5	-	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
24	48	27.50 ± 0.38^{b}	80.00 ± 0.09^{c}	0.00 ± 0.00^{b}				
	72	22.50 ± 0.72^{d}	77.5 ± 0.53^{d}	0.00 ± 0.00^{b}	6	+	Callusing	0.00 ± 0.00^{c}
	96	20.00 ± 0.62^{e}	72.5 ± 0.53^{e}	0.00 ± 0.00^{b}				
48	48	5.00 ± 0.85^{g}	95.00 ± 0.47^{a}	3.57 ± 0.07^{a}	7	++	4.00 ± 0.49^{b}	1.00 ± 0.19^{b}
	72	7.50 ± 0.40^{f}	87.50 ± 0.76^{b}	0.00 ± 0.00^{b}				
	96	7.50 ± 0.31^{f}	80.00 ± 0.09^{c}	0.00 ± 0.00^{b}	8	+++	Callusing	0.00 ± 0.00^{c}
$CD_{0.05}$		1.68	1.47	0.07			1.07	0.28

Bacterial overgrowth: - = Nil, + = Very less, ++ = More, +++ = Excess

efficient and resulted in 4.00 ± 0.49 average per cent shoot regeneration (Table 4, Fig 3A).

Formation of putative transgenic shoots: After optimizing the critical factors in apple rootstock M7, leaves were transformed with *A tumefaciens* strain LBA 4404 containing *rolB* and *nptII* marker genes and cultured on selective regeneration medium in order to ensure the induction of callus/shoots. It has been observed that only a few leaf explants induced greenish brown callus at certain areas and shoots directly as well as through callus after 50 days of co-culture on medium containing 0.6 mg/l TDZ and 0.5 mg/l NAA (Fig 3B).

DISCUSSION

Various parameters affecting gene delivery have been proven to be critical in the development of a successful transformation system. Several researchers found that young expanding leaves were the most effective explants for transformation experiments in various *Malus* cultivars and rootstocks (Zhu et al 2001, Yao et al 2013, Arcos et al 2020, Verma et al 2021). In the present study, morphogenesis could still be induced in small emerging leaves by increasing the amount of exogenous BA. Due to the poor development of vascular tissues, a higher concentration of BA was necessary at this stage. The current findings suggest that a high concentration of BA can lead to higher shoot regeneration which is consistent with the previous research (Sharma et al 2012). It indicated that higher concentration of cytokinin with lower auxin was required to increase regeneration rate in apple leaf explants (James et al 1984, Fasolo et al 1989).

The present findings demonstrated that MS medium having 4 mg/l BA with 0.5 mg/l NAA resulted in lower shoot regeneration rate as compared to 0.6 mg/l TDZ with same concentration of NAA suggesting that BA at higher level and TDZ at lower level increased the regeneration rate. It was found that NAA and IAA auxins were responsive to organogenesis with BA and TDZ, whereas, IBA did not regenerate shoots. Different varieties of Fuji induced callus formation on high concentration of TDZ ie 0.8 and 2.2 mg/l (Chen et al 2012, Arcos et al 2020). In the present study, some of the TDZ combinations resulted in vitrified and abnormal shoots while BA produced healthy and non-vitrified ones. On 1 mg/l TDZ and above hyperhydricity/ vitrification with rosette habit was identified in a number of apple genotypes (Wilson and James 2003).

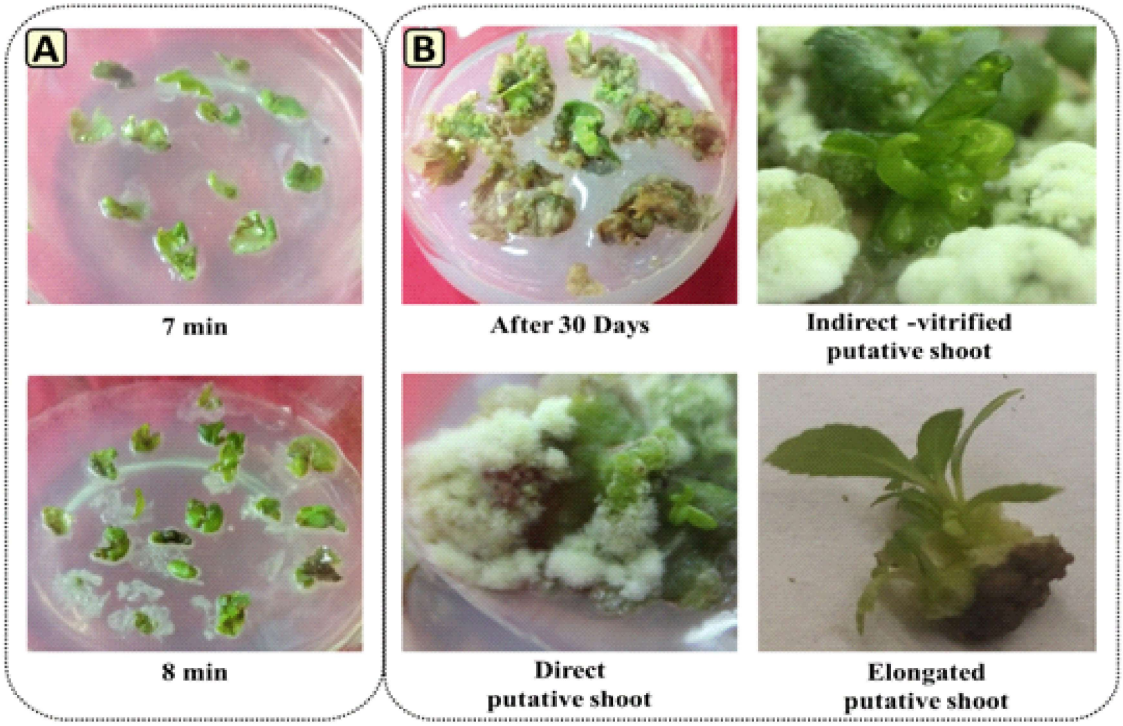

Fig 3. (A) Growth of *Agrobacterium tumefaciens* and explants survival with different infection times, (B) Leaf explants showing growth and formation of putative shoots after co-cultivation

Therefore, it is concluded that TDZ at specific level appeared to be more effective than BA for encouraging adventitious shoots from M7 leaves. Likewise, several studies showed that the TDZ was more effective for shoot regeneration than BA (Mitic et al 2012, Li et al 2014).

The concentration of kanamycin and cefotaxime has an inverse relationship with callus induction and shoot regeneration. Kanamycin concentration is the main factor that affects the explant survival and mortality rate. In the current experiments, callus induction was observed only at lower doses of kanamycin. Higher concentrations resulted in maximum explants death.

The results indicated that 35 mg/l kanamycin was a critical dose for putative shoot selection which differed from many other studies in apple (Yepes and Aldwinckle 1994, Dolgov et al 2000, Verma et al 2021). Similarly, the dose of cefotaxime affects shoot regeneration, contamination rate and mortality rate. In control experiments, shoot regeneration from callus was achieved at 200 and 300 mg/l cefotaxime while higher doses of 400 and 500 mg/l promoted callus.

In apple, strain selection is one of the most important factors influencing the efficiency of transformation. Several authors have utilized *A tumefaciens* strain LBA4404 in *Malus* species (Song et al 2000, Hanke et al 2001, Sharma et al 2017) because it can efficiently be eliminated from explants with low amount of antibiotics (Maheswaran et al 1992). In the present study, pre-cultivating leaves for 48 h and then co-cultivating in the dark for 48 h, promoted efficient infection. These results are supported by the work of Sangwan et al (1992) and Verma et al (2021) who found that pre-culturing period of two days was appropriate. Whereas, in apple cultivars Gala and Golden Delicious, pre-culturing period of 0-5 days had no effect on transformation efficiency (Yao et al 1995). These differences might be due to the different genotypes and type of explants used.

After co-cultivation with *Agrobacterium*, higher dose of 500 mg/l cefotaxime successfully inhibited bacterial growth, while decreasing it after some days resulted in shoot induction on selection medium. Similarly, in some previous reports, cefotaxime at 500 mg/l was found effective to eradicate the bacterial overgrowth (Sharma et al 2017, Verma et al 2021). Similar effects of cefotaxime have been shown

on leaf morphogenesis in pear and apple (Predieri et al 1989, Modgil and Sharma 2008).

Infection for longer duration promoted uncontrolled growth of *Agrobacterium* which resulted in necrosis of leaf explants, whereas, shorter duration exhibited negligible growth around leaves. Previous authors have reported best infection in 1 minute in *Malus domestica* cv Jonagold (De Bondt et al 1994) and 5 minutes in M26 (Maheswaran et al 1992). In other reports, fruit tree leaf explants infection time ranged from 5 to 120 minutes (Zhang et al 2006, Liu and Pijut 2010, Verma et al 2021). These results indicated that infection time is also affected by the genotype. Using these factors, the first putative transformed shoots were obtained 50 days after co-cultivation.

In conclusion, various factors prior to actual *Agrobacterium*-mediated M7 transformation trials have been standardized. In this study, pre-culturing of leaf explants for 48 h, infection for 7 min and co-cultivation for 48 h on medium containing lower doses of TDZ and NAA supplemented with 500 mg/l cefotaxime and 35 mg/l kanamycin, maximized the rate of undamaged leaf explants.

ACKNOWLEDGEMENT

Financial assistance received from ICAR – Central Assistance Scheme provided by the Dean, College of Horticulture, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh is highly acknowledged.

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