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Optimizing *In Vitro* Regeneration from Iranian Native Dwarf Rootstock of Apple (*Malus domestica* Borkh)

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ABSTRACT

The regeneration potential is different among cultivars and must be optimized for individual genotypes. Regeneration of adventitious shoots from native dwarf rootstock of apple (*Malus domestica* Borkh cv Gami Almasi) was optimized for the first time. Shoot regeneration step is a critical factor in apple *Agrobacterium* mediated transformation. In this experiment a protocol for direct organogenesis from leaf of *in vitro* grown shoots obtained from apple CV Gami Almasi. Two regeneration medium with MS, N6 compounds and various combinations of BA and NAA were used. Also two leaf and callus tissue used for regeneration tests. MS medium was better than N6 for shoot regeneration and optimum phytohormons concentrations were achieved in 7.5 mg L^{-1} BA and 2 mg L^{-1} NAA. Shoots that regenerated were obtained only from leaf and callus did not regenerate any shoot. Shoot usually regenerated without callus from leaves. Highest percentage of shoot regeneration from leaf with 93.75% in MS medium was achieved.

Key Word: Apple; Dwarf rootstock; Gami almasi; Regeneration

INTRODUCTION

Increasing regeneration efficiency is critical for the development of transformation system in apple using *Agrobacterium tumefaciens* vectors. (Klee & Rogers, 1989) In many instances, the lack of efficient regeneration systems is the major limiting factors that prevent the development of gene transfer technologies for perennial crops (Dandekar, 1992). For this purpose, leaf and callus explants from apple plants grown *in vitro* have often been used. Leaf tissue contains no pre-existing shoot primordia and the tissue is more likely to produce non-chimeral plants. First report of *in vitro* regeneration of adventitious shoots from apple leaf tissue was provided by Liu *et al*. (1983). Several later reports revealed critical factors affecting the frequency of leaf regeneration in apple scion cultivars, rootstocks and seed explants (James *et al.,* 1984; 1988; Barbieri & Morini, 1987; Welander, 1988; Fasolo *et al*., 1989; Dufour, 1990; Korban *et al*., 1992; Sriskandarajah *et al*., 1981, 1982, 1998). Most apple cultivars are recalcitrant in terms of adventitious organ formation. "Gami Almasi" is a dwarf rootstock that can be used for different scion cultivars of apple. Also efficient regeneration has not been demonstrated yet for this rootstock. The objective of this work was to optimize regeneration frequency from leaf and callus with the final goal of enhancing recovery of transformed apple plants. The effect on apple organogenesis of several concentrations of BA and NAA that are used during regeneration was studied, because previous reports revealed that optimum concentration of BA and NAA different among genotypes (Yepes & Aldwinckle, 1994). Apple leaves regenerated *in vitro* are influenced by many factors (Liu *et al*., 1983; Fasolo *et al*., 1989). Optimum concentrations of growth regulators and best culture medium were achieved for different cultivars and rootstock; also pretreatment and leaf orientation in contact with medium were tested (Korban *et al*., 1992; Sriskandarajah *et al*., 1998).

MATERIALS AND METHODS

Plant material. Shoots were excised aseptically from *in vitro* grown cultures, of the apple rootstock cultivar "Gami Almasi" and proliferated in MS (Murashige & Skoog) medium contain 10 mg L^{-1} BA and 1 mg L^{-1} IBA before use in regeneration experiments. Callus derived from *in vitro* stem, propagated in MS medium contained 10 mg L^{-1} NAA before use in regeneration experiment. Severed 5 ± 2 mm segments from callus and culture in petridishes. For leaves each unfurled leaf length 15 ± 3 mm were excised from the shoots and then wounding by scalper and placed from abaxial side touching the medium. The media used were MS and N6 (Modified by Welander, 1988). Compositions of the basal media that were tested are listed in Table I. Growth regulators that used were 0, 2.5, 5, 7.5, 10 mg L^{-1} BA in combination with 0, 0.2, 1 and 2 mg L^{-1} NAA. The pH of the medium was adjusted to 5.8 with 0.1 NaOH prior to adding agar. All media were autoclaved for 20 min at 121°C and 1.1 kg cm^{-2} in flasks and then dispensed aseptically in 60 × 15 mm sterile plastic petridish. Then leaves were

Table I. Component of basal media (mg L^{-1})

Components	N6 (Modified by Welander 1988)	MS (Murashig & Skoog)
NH4NO3	-	1650
(NH4) 2SO4	463	-
KNO3	2830	1900
CaCl2.2H2O	166	440
MgSO4	185	370
KH2PO4	400	170
Na2EDTA	37.3	37.3
FeSO4	27.8	27.8
MnSO4.4H2O	22.3	22.3
ZnSO4.7H2O	8.6	8.6
H3BO3	6.2	6.2
KI	0.83	0.83
Na2MoO4.2H2O	0.25	0.25
CuSO4.5H2O	0.025	0.025
CoCl2.6H2O	0.025	0.025
Vitamins		
Niconitic acid	0.5	0.5
Thiamin.HCl	0.1	0.1
Pyrodoxine	0.5	0.5
Glycine	2.0	2.0
Myo_inositol	100	100
Others		
Sucrose	30,000	30,000
Agar_Agar	7000	7000

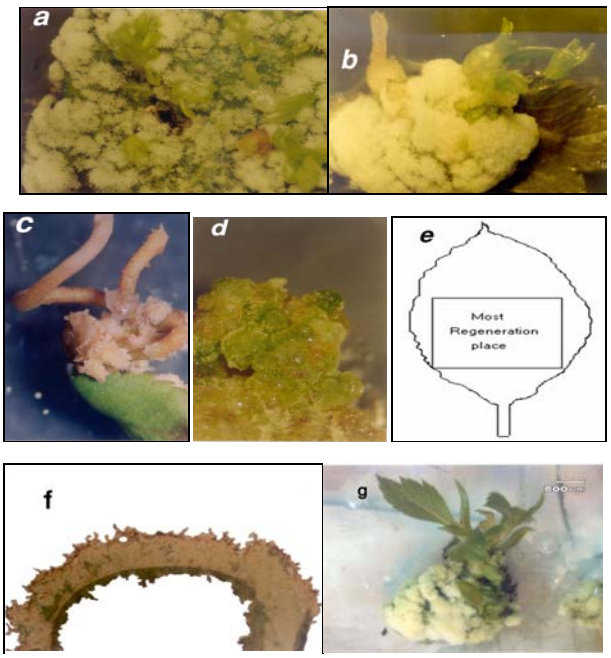
explanted on the medium the dishes were sealed with parafilm. The cultures were placed in dark at 25 ± 0.5°C for the 10 days and when moved to the light (4500 lux), the conditions were 25 ± 2°C with 16/8 h photoperiod provided by warm and cool white fluorescent tubes. Factors that were tested in these experiments were two different culture media, cytokinin and auxin concentration and tissue type.

Experimental design and data. Four replicate each containing four leaves or callus segments in petridishes were used in randomized complete block design for all experiments. The number of leaves forming shoots and number of shoots formed per leaf were recorded. Also percentage of callus inducted leaves was counted. Data was recorded 45 days after the start of the experiment. Data were analyzed statistically using MSTATC software. The Duncan test was applied for means separation at $P < 0.01$.

RESULTS

Using a dissecting microscope adventitious buds and shoots were observed 20 day after plating (Fig. 1a) Adventitious shoots developed as nodules with a glossy surface as compared with more diffuse appearance of other filamentous cells that occurred simultaneously along the edge and surface of the leaf. The number of shoots continued to increase during following six to eight weeks. Morphogenesis occurred mostly on the wounding position and center of leaf blade near the basal leaf (Fig. 1e). Adventitious shoots usually developed without the presence of callus (Fig. 1a, b). Leaves produce regenerants for up to 3 month. During this time, the shoot mass increased substantially in size and weight until the number of

Fig. 1a Shoots regenerated from leaf surface directly, (b) shoot regenerated from callus mediated from leaf surface, (c) Root formation from edge of leaf in absence of cytokinin, (d) callus without regeneration, (e) position of most regenerative segment on leaf, (f) profile of leaf after callus induction, (g) shoots after 6 weeks from regeneration

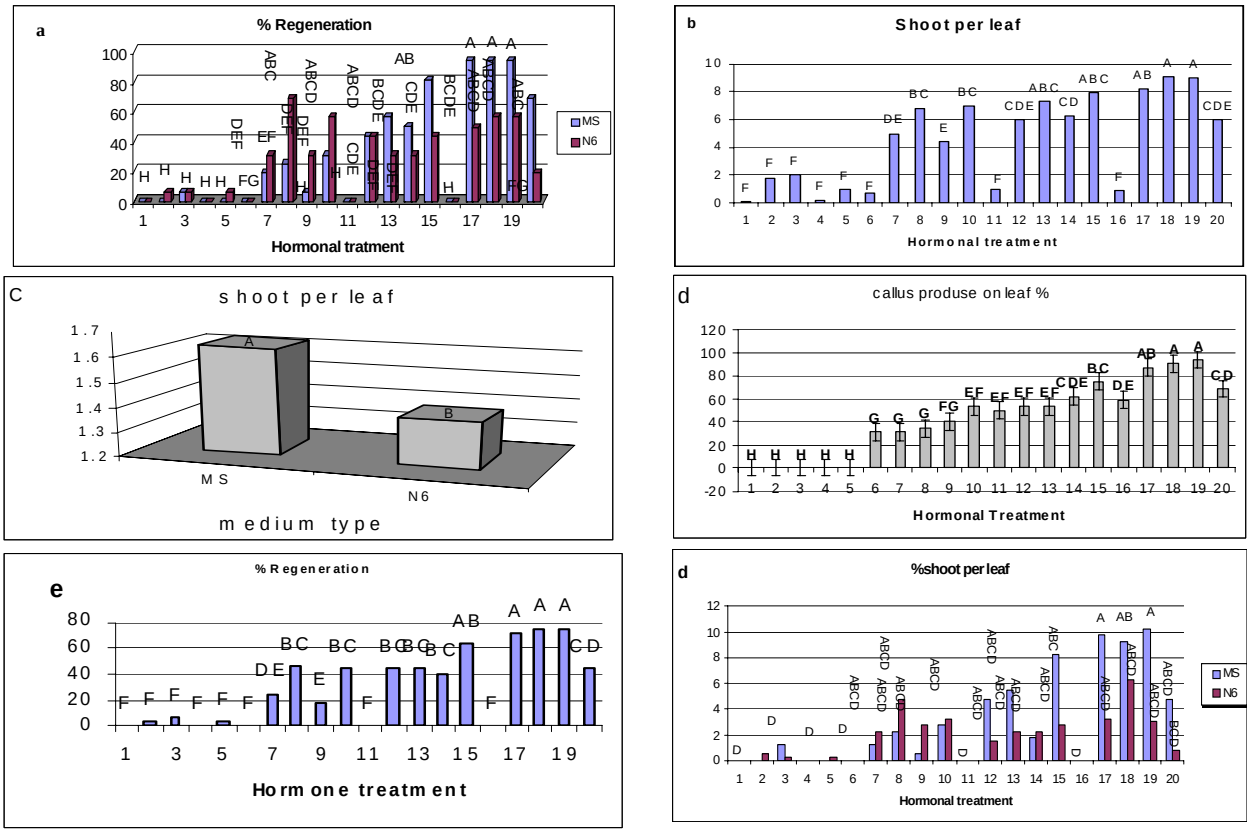


regenerants could not be counted during second month. In control plants, in which no growth regulator were added to medium did not regenerate any shoot from leaves. In treatments 6, 11, 16 to which BA was added, root formation occurred at edge and midrib of leaf (Fig. 1c).

Effect of regeneration media with BA and NAA levels. There were combination of different cytokinin and auxin concentration (Table II). The combined effect of two different basal media with cytokinin and auxin concentrations on regeneration percentage, shoot formation per leaf and callus formation percentage are given in Fig. 2a, b, c, d, e & f. Analysis of variance indicated that interaction medium with auxin and cytokinin concentrations were significant. The highest regeneration percentages for leaf 93.75 were obtained for 17, 18 and 19 treatments on MS medium (Fig. 2a, e), but this percentage was not significant between MS and N6 medium. Also the highest mean of shoot per leaf, was obtained in this concentration of BA and NAA (Fig. 2b, f). Increased concentration of BA and NAA increased both regeneration percentage and mean number of shoots regenerated per leaf (Fig. 2a, c).

The relationship between these two response variables and the BA level was not totality linear, because at higher concentration of BA (10 mg L^{-1}) the regeneration percentage was reduced. In comparison between two MS and N6 medium, the MS medium was better than N6 for

Fig. 2a. Inter action effect of different hormonal treatment with MS and N6 medium on regeneration percentage, (b) the hormonal treatment effect on shoot regeneration per leaf, (c) medium effect on shoot regeneration per leaf, (d) percentage of explants that produce callus on leaf surface, (e) regeneration percentage on differet hormonal combinations, (f) interaction effect between media and different hormonal combinitions on number of shoot regeneration per leaf



number of shoot per leaf (P = 0.01) but no significant effect on regeneration percentage and callus induction percentage (Fig. 1c). Interaction between media and hormone concentration were significant (P < 0.01). Although hormone concentration was the important single factor for regeneration (highest F value), low concentration of auxin was needed for optimum regeneration. BA alone (without NAA) had no effect on regeneration. Of the two tissue types (callus and leaf), no shoot formation were observed on the callus. Callus growth on media was similar to regeneration primordia but did not produce any shoot over three month duration (Fig. 1d). Callus in treatment 8, 12, 13, 17, 18, 19, 20 showed the best growth. Callus induction on surface of leaf recorded for each treatment. The hormone effects on callus induction percentage were significant (Fig. 2d) but the medium type had no effect on callus induction of leaf. Callus formation and shoot regeneration were observed earlier in N6 than MS.

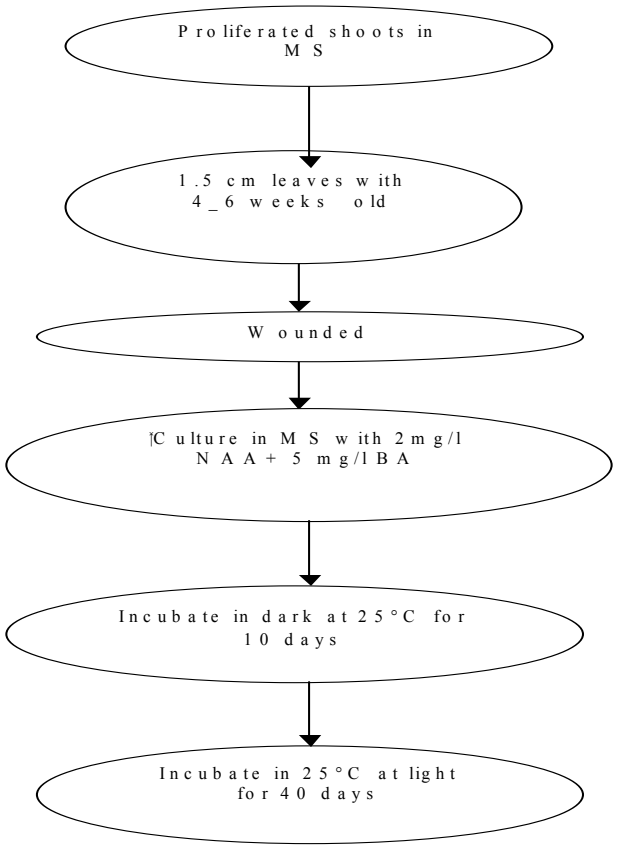
DISCUSSION

In this study the regeneration of adventitious shoot were formed only in leaf tissue and callus did not regenerate any shoot in all treatments. In regenerated leaf that, formation of adventitious shoots regenerated usually occurred without an intermediate callus stage, but shoot formation with callus intermediate in low frequency were sometimes achieved. Adventitious regeneration in plant occurs after from organized tissues dedifferentiate and then reorganized by meristem or somatic embryo formation, either directly or indirectly with an intermediate callus stage (Litz & Gray, 1992). The main factor affecting morphogenesis in our study, were tissue type hormone level (BA & NAA concentration) and medium composition, respectively. A high cytokinin:low auxin ratio was required for promoting regeneration in apple leaf explants (Fasolo *et al.*, 1989; James *et al.*, 1984, 1988; Welander, 1988). In our study optimal BA and NAA concentrations to induce regeneration of "Gami Almasi" were 5 and 2 mg L^{-1},

Table II. Number of different hormonal treatment

10 mg L^{-1} BA	7.5mg L^{-1} BA	5 mg L^{-1} BA	2.5mg L^{-1} BA	0 mg L^{-1} BA	
5	4	3	2	1	0 mg L^{-1} NAA
10	9	8	7	6	0.2 mg L^{-1} NAA
15	14	13	12	11	1.0 mg L^{-1} NAA
20	19	18	17	16	2.0 mg L^{-1} NAA

Fig. 3. Regeneration protocol from "Gami Almasi" variety



respectively. N6 and B5 medium contain lesser ammonium than MS. Welander (1988) reported that a medium with reduced ammonium promoted the regeneration from apple. It seemed that regeneration response to low concentration of ammonium was dependent upon genotype; and some genotype of apple did not respond to low ammonium. Yeps and Aldwinckle showed that medium effect on regeneration of different apple genotypes and some genotypes such as "Golden delicious" regenerated on MS medium better than N6 (Yeps & aldwinckle, 1994). The MS medium stimulated morphogenesis more than N6 medium for this rootstock of apple. It is likely high concentrations of $CaCl_2$ $2H_2O$ and $MgSO_4.7H_2O$ in MS than N6 had effect on regeneration from leaf.

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