Pak. J. Bot., 42(6): 3817-3830, 2010.

DIRECT PLANT REGENERATION FROM LEAVES OF PRUNUS ROOTSTOCK GF-677 (*PRUNUS AMYGDALUS* X *P. PERSICA*)

SYED ZIA UL HASAN, TOUQEER AHMAD, ISHFAQ AHMAD HAFIZ* AND AZHAR HUSSAIN

Department of Horticulture,
Pir Mehr Ali Shah Arid Agriculture, University, Rawalpindi, Pakistan
**Corresponding author E-mail:*

Abstract

The effect of two culture media *viz*., Murashige & Skoog (MS) and Lepoivre (LP) supplemented with 1.0, 1.5, 2.0, 2.5 mg l^{-1} benzylaminopurine (BAP) and 0.25, 0.5, 0.75, 1.0 mg l^{-1} naphthalene acetic acid (NAA) was examined on shoot regeneration from leaves of *Prunus* rootstock GF-677. LP medium was found to be superior producing 29.85% regenerated shoot, 3.39 shoot number and 3.43 cm shoot length whereas MS medium produced 20.88% regenerated shoot, 1.90 shoot number and 2.28 cm shoot length. Moreover, LP medium produced longer and healthy shoots whereas stunted and yellow shoots were observed on MS medium. Regarding the interaction between media and growth regulators, the best interaction was found in LP medium supplemented with 2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA (T_{11}) resulting in 55% regenerated shoots, 4.28 shoot number and 4.25 cm shoot length while 40% regenerated shoots, 3.20 numbers of shoots and 3.80 cm shoot length were observed on MS medium with the same combination of cytokinins and auxins. As far as rooting is concerned, the effect of two iron chelating agents *viz*., ethylenediamine di-o-hydroxyphenylacetic acid (Fe-EDDHA) and ethylenediamine tetraacetic acid (Fe-EDTA) was evaluated at concentration of 100, 200 and 300 mg l^{-1}. Fe-EDDHA proved to be superior chelating agent producing 95% rooting percentage, 3.40 root number and 6.88 cm root length at 200 mg l^{-1} (T_5). Conversely Fe-EDTA produced about 40% rooting percentage, 1.83 root number and 2.57 cm root length at 100 mg l^{-1} (T_1). Chlorotic and small roots were observed with Fe-EDTA while strong and larger roots were obtained with Fe-EDDHA.

Introduction

GF-677 (*Prunus amygdalus* x *P. persica*) has proved to be potential rootstock for prunus species because it is well adapted to poor soil fertility and drought conditions (Monticelli *et al*., 2000). However it produces highly vigorous tree and hence cannot fulfill the requirement for recent trends of high density planting system. Moreover, rooting percentage is also very poor (Ahmad, 2002). The high density planting require dwarf rootstocks. Ideal dwarfing rootstock should have good compatibility with scion cultivars, dwarfing potential, high rooting ability and freedom from nursery diseases as well as good adaptation to adverse environmental condition (Zhu *et al*., 2003). Therefore, it is of great importance to select new or improve the existing rootstocks.

Conventional breeding of woody fruit species is a slow and difficult process due to elevated levels of heterozygosity and extended regeneration cycles (Sriskandarajah *et al*., 1994). Consequently, it is important to develop gene-transfer methods for fruit crops, to accelerate the breeding process and enlarge the germplasm resources available for crop improvement (Ainsley *et al*., 2000). By using genetic transformation method, the breeding process can be greatly accelerated (Zhu *et al*., 2003). The transformation of plants with *rol* genes has been reported to show great agronomic value such as dwarf

plants with shorter and numerous shoots, compact vegetative habit, large root systems and high rooting ability of the cuttings (Rugini & Pesce, 2006). Transformation of GF-677 rootstock with *rol* gene may help to produce dwarf plants with extensive root system which are suitable for high density planting. A good regeneration protocol is a prerequisite for successful gene transfer *via Agrobacterium* (Zhu *et al*., 2003). Moreover, Zhu *et al*., (2003) also reported that it is generally difficult to regenerate adult material from woody species and the regeneration frequency is much genotype dependent. It is necessary to optimize conditions to identify regenerability of a given genotype. So the present studies were designed to develop an efficient and effective regeneration protocol for GF-677 rootstock which would be useful in future research work regarding genetic transformation of this economically important rootstock.

Materials and Methods

Leaves of GF-677 *Prunes* rootstock maintained on shoot proliferation media (Ahmad *et al*., 2003) and subcultured every three weeks were used as an experimental material. First four (20 days old) fully expanded leaves were excised. Each leaf was wounded by three to four cuts transversely to the midrib, without full separation and placed with adaxial surface in contact with the regeneration media in culture jars. For the shoot regeneration study two culture media *viz*., MS (MS macro and micro elements) medium (Murashige & Skoog, 1962) and LP (LP macro and micro elements) medium (Quoirin *et al*., 1977) with different growth regulators combination were tested (Table 1). Comparison of both media is given in Table 2. Both the media were supplemented with MS vitamins, 100 mg l^{-1} myo-inositol, 30 g l^{-1} sucrose and 7.0 g l^{-1} agar. The pH of both media was adjusted to 5.8 before autoclaving. The explants were maintained at 25 ± 1ºC for 21 days in dark and then transferred to light under 16/8 hr photoperiod (2,000 lux) with white fluorescent tubes (Philips TL 40W/54). The experiment was bifactorial (Media x Growth regulators) randomized in CRD (completely randomized design) with four replication per treatment and ten explants per replication. Data was recorded after four weeks regarding shoot regeneration percentage, number of shoots per regenerated explant and length (cm) of the regenerated shoots.

For rooting study uniform sized shoots (1-1.5 cm) were transferred to rooting medium (half strength MS macro and micro elements) supplemented with MS vitamins, 100 mg l^{-1} myo-inositol, 0. 4 mg l^{-1} IBA, 0. 2 mg l^{-1} IAA, 20 g l^{-1} glucose and 7 g l^{-1} agar (Ahmad *et al*., 2004). The effect of two chelated forms of iron salts *viz.*, ethylenediamine di-o-hydroxyphenylacetic acid (Fe-EDDHA) and ethylenediamine tetraacetic acid (Fe-EDTA) at the concentration of 100, 200 and 300 mg l^{-1} on *In vi*tro rooting of GF-677 rootstock was examined. The pH of the culture medium was adjusted to 5.8 before autoclaving. Cultures were incubated at 25 ± 1ºC under 16/8 hr photoperiod (2,000 lux) with white fluorescent tubes (Philips TL 40W/54).The experiment was laid out in CRD using four replication per treatment and ten shoots per replication. Data was recorded after four weeks regarding rooting percentage, number of roots per explant and root length (cm). Statistical analysis of the data was carried out by using Analysis of Variance (ANOVA) technique and difference among treatment means were compared by using Least Significance Difference Test (LSD) at 5% probability level (Steel *et al*., 1997).

Table 1. Different media composition (MS and LP) and growth regulators (BAP and NAA) for *In vitro* shoot regeneration of *Prunus* rootstock GF 677.

Treatments	MS (MS macro and micro elements) Medium		LP (LP macro and micro elements) Medium	
	BAP mg l^{-1}	NAA mg l^{-1}	BAP mg l^{-1}	NAA mg l^{-1}
T_0	0.0	0.0	0.0	0.0
T_1	1.0	0.25	1.0	0.25
T_2	1.0	0.50	1.0	0.50
T_3	1.0	0.75	1.0	0.75
T_4	1.0	1.0	1.0	1.0
T_5	1.5	0.25	1.5	0.25
T_6	1.5	0.50	1.5	0.50
T_7	1.5	0.75	1.5	0.75
T_8	1.5	1.0	1.5	1.0
T_9	2.0	0.25	2.0	0.25
T_{10}	2.0	0.50	2.0	0.50
T_{11}	2.0	0.75	2.0	0.75
T_{12}	2.0	1.0	2.0	1.0
T_{13}	2.5	0.25	2.5	0.25
T_{14}	2.5	0.50	2.5	0.50
T_{15}	2.5	0.75	2.5	0.75
T_{16}	2.5	1.0	2.5	1.0

Table 2. Comparison of mineral elements of MS and LP medium.

Mineral elements	MS mg l^{-1}	LP mg l^{-1}
N	844.05	535.09
P	38.75	61.54
K	782.95	772.49
Ca	149.15	203.38
Mg	36.09	35.12
I	0.635	0.061
S	55.49	51.12
Cl	261.01	0.0073
Fe	5.61	5.61
Na	5.268	5.268
B	1.1	1.10
Zn	1.94	1.94
Mo	0.119	0.119
Cu	0.0064	0.0064
Co	0.0062	0.0062

Results and Discussion

Regeneration percentage: Both culture media (MS and LP) have a pronounced effect on the regeneration percentage (Table 3). A significantly higher mean shoot regeneration percentage was observed on LP medium (29.85) as compared to MS medium (20.88). The better results obtained in LP medium as compared to MS medium might be due to difference in ammonium to nitrate ratio. The ammonium to nitrate proportion is 1.9: 1 in MS medium while 1: 1.6 in LP medium. Thrope *et al*., (1989) reported that optimum ammonium to nitrate ratio for adventitious shoot formation in *Picea glauca* was around 1:3. Nitrate and ammonium salt are main sources of nitrogen in the culture medium. Nitrate is a better source of nitrogen because of its ability to be readily taken up, better assimilation and non-toxic nature as compared to ammonium source (Shanjani, 2003; George *et al*., 2008). Igarashi & Yuasa (1994) reported that the regeneration frequencies can be improved significantly by reducing the total amount of nitrogen and ratio of NH_4 to NO_3 in the media.

A significant interaction between media and growth regulators regarding the shoot regeneration percentage was found at $p<0.05$. The shoot regeneration percentage was 55% on LP medium supplemented with 2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA as compared to 40% on MS medium with same combination of auxin and cytokinins (Table 3). Ali *et al*., (2009) reported that influence of growth regulators can be modified by the culture medium. On both (LP and MS) media an increasing trend in regeneration percentage was obtained upto T_{11} (2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA) after which the shoot regeneration percentage decreased gradually. Ali *et al*., (2009) reported that the optimum combination of cytokinins and auxin is critical to shoot regeneration.

The shoot regeneration percentage was significantly affected by treatments. Maximum shoot regeneration percentage (47.50) was obtained with T_{11} (2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA) as compared to other combinations of BAP and NAA (Table 3). BAP in combination with NAA has enhanced shoot regeneration percentage in both sweet and sour cherry cultivars (James, 1987). A low frequency of differentiation was obtained in lychee explants when cytokinins were added singly in the regeneration media (Puchooa, 2004). George *et al*., (2008) reported that cytokinins when added with auxins promote cell division. Nordstrom *et al*., (2004) suggested that auxins may also play a direct regulatory role in the balance of cytokinins levels by suppressing both the synthesis rate and pool size of cytokinins.

Number of shoots per regenerated explant: Statistical analysis revealed that LP medium proved to be better than MS medium for the number of shoots per regenerated explant (Table 3). Maximum number of shoots was observed on LP medium (2.39) which was significantly higher than that produced by MS medium (1.90). The better results obtained in LP medium might be due to the contribution of calcium in form of $CaNO_3$ instead of $CaCl_2$ composing the MS medium. The proportion of calcium between MS and LP medium is around 1:1.4. Lamrioui *et al*., (2009) reported the beneficial effects of replacing $CaCl_2$ with $CaNO_3$ in micropropagation of wild cherry. George *et al*., (2008) reported that chloride toxicity can result if too much calcium chloride is added to the medium. The poor results obtained on MS medium might be due to much higher contents of chloride as compared to LP medium. The proportion of chloride between LP and MS medium is around 1: 35753. George *et al*., (2008) found that higher concentration of chloride caused woody species to have yellow leaves and weak stems while sometimes tissues collapsed and died.

Table 3. Effects of different culture media (MS and LP) and growth regulators (BAP and NAA) on regeneration percentage, number and length of regenerated shoots of *Prunus* rootstock GF- 677.

Treatments BAP and NAA mg l^{-1}		Regeneration % age		Mean	Number of shoot per regenerated explant		Mean	Length (cm) of the regenerated shoots		Mean
		MS	LP		MS	LP		MS	LP	
T_0	0.0 + 0.0	0.00 m	0.00 m	0.00 I	0.00 r	0.00 r	0.00 J	0.00 q	0.00 q	0.00 K
T_1	1.0 + 0.25	10.00 l	15.00 jkl	12.50 H	1.00 q	1.50 o	1.25 I	1.28 p	1.55 no	1.41 J
T_2	1.0 + 0.5	12.50 kl	17.50 ijkl	15.00 H	1.25 p	1.88 klm	1.56 H	1.55 no	1.83 lm	1.69 I
T_3	1.0 + 0.75	12.50 kl	20.00 hijk	16.25 GH	1.10 q	1.90 k	1.50 H	1.28 p	1.88 lm	1.58 I
T_4	1.0 + 1.0	10.00 l	15.00 jkl	12.50 H	1.75 lmn	1.98 k	1.86 G	1.45 op	1.70 mn	1.58 I
T_5	1.5 + 0.25	20.00 hijk	30.00 defg	25.00 EF	1.88 klm	2.30 hij	2.09 F	2.15 jk	2.20 ij	2.18 G
T_6	1.5 + 0.5	22.50 ghij	35.00 cde	28.75 DE	2.30 hij	2.43 gh	2.36 DE	2.30 ij	2.55 gh	4.43 F
T_7	1.5 + 0.75	25.00 fghi	40.00 bc	32.50 CD	2.35 hij	2.53 g	2.44 D	2.63 g	2.63 g	2.63 E
T_8	1.5 + 1.0	27.50 efgh	32.50 cdef	28.75 DE	2.25 ij	2.40 ghi	2.33 E	2.83 f	2.95 f	2.89 D
T_9	2.0 + 0.25	30.00 defg	40.00 bc	35.00 BC	3.00 e	3.20 d	3.10 C	3.40 de	3.38 e	2.39 C
T_{10}	2.0 + 0.5	35.00 cde	40.00 bc	40.00 B	3.08 de	3.50 c	3.29 B	3.60 bcd	3.62 bc	3.61 B
T_{11}	2.0 + 0.75	40.00 bc	55.00 a	47.50 A	3.20 d	4.28 a	3.74 A	3.80 b	4.25 a	4.03 A
T_{12}	2.0 + 1.0	32.50 cdef	45.00 b	38.75 B	2.80 f	3.83 b	3.13 B	3.45 cde	3.68 b	3.56 B
T_{13}	2.5 + 0.25	20.00 hijk	37.50 bcd	31.25 CD	1.85 klm	2.70 f	2.28 E	2.38 hi	2.90 f	2.64 E
T_{14}	2.5 + 0.5	20.00 hijk	32.50 cdef	27.50 DE	1.68 n	2.23 j	1.95 G	2.28 ij	2.55 gh	2.41 F
T_{15}	2.5 + 0.75	22.50 ghij	27.50 efgh	25.00 EF	1.38 op	2.33 hij	1.85 G	2.15 jk	1.96 kl	2.05 GH
T_{16}	2.5 + 1.0	15.00 jkl	25.00 fghi	21.25 G	1.37 op	1.72 mn	1.55 H	2.18 ij	1.70 mn	1.94 H
	Mean	20.88 B	29.85 A		1.90 B	2.39 A		2.28 B	2.43 A	
	LSD 5%	Medium	Interaction (MxT)	Treatments	Medium	Interaction (MxT)	Treatments	Medium	Interaction (MxT)	Treatments
		9.470	6.696	4.735	0.2080	0.1471	0.1040	0.2734	0.1933	0.1367

Fig. 1. Maximum number of shoots in (a) LP medium (b) MS medium with 2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA (T_{11}).

Fig. 2. Reduced number of shoots in (a) LP medium (b) MS medium at T_{12} (2.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA).

Significant interaction was found between media and growth regulator at $p< 0.05$ for number of shoots per regenerated explant (Table 3). The combination of 2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA (T_{11}) showed better interaction with both LP and MS medium producing 4.28 and 3.20 shoots per regenerated explant respectively (Fig. 1a & b). Skoog & Miller (1957) suggested that shoot formation could be induced from tobacco callus using high level of cytokinins with relatively low level of auxin in the growth media. The positive interaction between salts of LP medium and growth regulators might be attributed to higher calcium contents as compared to MS medium. Ali *et al*., (2009) found that increase in the internal concentration of calcium of cytokinins treated cells of olive explants led to more cell division. In both MS and LP medium an increasing trend in number of shoots per explants was observed upto T_{11} (2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA). However a declining trend is observed by increasing the concentration of BAP to 2.0 mg l^{-1} along with 1.0 mg l^{-1} NAA (Fig. 2a & b). Welander (1988) reported that high cytokinin to auxins ratio was required for promoting regeneration in apple leaf explants suggesting that at higher concentration there is a drastic decrease in number of shoots per regenerated explant in both MS and LP medium. It is obvious from the present study that the inclusion of NAA in the medium is a critical factor for enhancement of shoot regeneration.

The shoot number was significantly influenced by cytokinins and auxins combination. In the present study the combination of 2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA (T_{11}) showed synergetic effects producing maximum number of shoots (3.74) as compared to other treatments containing higher or lower concentration of BAP and NAA (Table 3). These results can be related to the manipulation of exogenous cytokinins concentrations that may cause an increase of endogenous auxin concentrations which is probably induced by an inhibition of free IAA conjugates due to the presence of exogenous cytokinins (Jaramillo *et al*., 2008). George *et al*., (2008) observed that the auxin to cytokinins ratio represents an important signal in the formation of cell phenotype and also in the onset and maintenance of the process of cell division. Since auxins are capable of imitating cell division they are involved in the formation of meristems giving rise to either unorganized tissue or defined organs (George *et al*., 2008).

Length of regenerated shoot (cm): The results indicated that both media differ significantly in terms of length of regenerated shoots. It is clearly visible from the data that best results obtained with LP medium showed the consistent trend as shown by the previous parameters of shoot number and regeneration percentage (Table 3). LP medium showed better results with respect to shoot length (2.43 cm) as compared to the MS medium (2.28 cm).

Statistical analysis showed significant interaction between the two media (MS and LP) and the growth regulators (BAP and NAA) regarding the shoot length (Table 3). As seen for the previous parameter of number of shoots the same trend was followed in the length of the regenerated shoots. The better response was showed at T_{11} (2.0 mg l^{-1} BAP and 0.75 mg l^{-1}) with both media producing 4.25 cm and 3.80 cm long shoots on LP and MS medium respectively (Fig. 3a & b). It has been reported that optimal nutrient composition may affect the sensitivity of explants in response to plant growth regulators (Kothari *et al*., 2004). Williams (1995) suggested that a limited addition of an essential element is one of the possible factors for limited plant growth. The optimum phosphorous level in LP medium might be responsible for better shoot elongation than MS medium. Kothari *et al*., (2004) suggested that the optimal nutrient composition in the medium affects the sensitivity of plants in response to plant growth regulators. A low supply of phosphorous may decrease cytokinins levels in the shoots and consequently decrease protein synthesis (De Groot *et al*., 2003). Murai (1994) argued cytokinins might regulate the biosynthesis of cytokinins active ribonucleoside in the tRNA modulating the concentration of particular subset of tRNA and thus affecting the rate of protein synthesis.

Ruzic *et al*., (2001) reported that phosphorous is not only the element which plant taken in the greatest amount and most rapidly but also its concentration in the media is of great importance as well. George *et al*., (2008) suggested depletion of phosphorous early during culture has a major effect on the pH of the medium in which phosphorus is a major buffering component. Kothari *et al*., (2004) reported that the growth rate of *In vitro* cultured plants is a function of net assimilation rate resulting from the uptake of nutrients from the media. PGR can partially compensate for the nutritional imbalances and by correcting the imbalance they can be reduced or eliminated as Gomez & Segura (1994) observed for NAA regarding shoot regeneration of junipers. Yu *et al*., (2001) found that effect of NAA is probably associated with increased cell number

Fig. 3. Maximum shoot length observed on (a) LP medium and (b) MS medium with 2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA (T_{11})

Fig. 4. Stunted shoot growth in (a) LP medium (b) MS medium with 2.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA (T_{12})

A linear relationship was observed with increasing the NAA concentration in relation to the BAP concentration. A declining trend was observed in the shoot length per regenerated explant after T_{11} (2.0 mg l^{-1} BAP and 0.75 mg l^{-1}) suggesting that at higher concentration the auxin and cytokinins have an inhibitory effect on shoot elongation (Fig. 4 a & b). Shirin *et al*., (2007) reported that meristomatic cell division in potato callus was blocked at high concentration of NAA which leads toward the death of the cells. Sajid *et al*., (2006) reported that presence of NAA in the medium had a positive effect on shoot length in the presence of BAP. It can be concluded from the results that higher concentration of cytokinins and auxins shows poor interaction with salts of both the media causing a decrease in the shoot length.

George *et al*., (2008) reported that cytokinins together with auxins play an important role in cell cycle regulation. In the present study the combination of BAP and NAA at T_{11} (2.0 mg l^{-1} BAP and 0.75 mg l^{-1} NAA) was found to be most optimum producing maximum shoot length (4.03 cm). George *et al*., (2008) suggested that cell division is regulated by joint action of cytokinins and auxins, each of which appears to influence different phases of cell cycle. Pasternak *et al*., (2000) reported that auxins exert an effect on DNA replication while cytokinins regulate the events relating to cell division. Higher concentration of auxin almost invariably increases ethylene production. Ethylene accumulated in tissue culture vessel inhibits growth and development of tissue culture plants (George *et al*., 2008; Yaseen *et al*., 2009).

Rooting percentage: Different levels of Fe-EDTA and Fe-EDDHA varied significantly regarding the rooting percentage (Table 4). Highest rooting percentage (95%) in Fe-EDDHA was found at T_5 (200 mg l^{-1}) whereas Fe-EDTA produced rooting in only 40 % of the shoots at T_1 (100 mg l^{-1}). The control treatments deprived of iron did not form roots which show that iron is an essential nutrient in plant tissue culture media as observed by Antonopoulou *et al*., (2007). It is reported to be involved in fundamental processes such as chlorophyll and DNA synthesis (George *et al*., 2008). According to Molassiotis *et al*., (2004) DNA synthesis is prerequisite for cell division which is one of the three basic processes of root formation. It is clear from the results that rooting percentage correlate with the concentration of chelating agents. In the present study Fe- EDDHA was found superior to Fe- EDTA regarding the rooting percentage. Vander Slam *et al*., (1994) reported that Fe-EDTA is less stable than Fe-EDDHA due to its susceptibility to light, its precipitation with KH_2PO_4 or with other micronutrient element compound. Antonopoulou *et al*., (2007) reported that the replacement of Fe-EDTA with Fe-EDDHA has positive influence on the micropropagation of various species. Antonopoulou *et al*., (2007) also found that the concentration of 37.3 mg l^{-1} Na_2EDTA is in excess to chelate 27.8 mg l^{-1} Fe^{2+}. They suggested that excessive quantity may affect availability of other divalent micronutrient cations. Hangarter & Stasinopoulos (1991) also found the formation of toxic formaldehyde after Fe-EDTA photo degradation. Fe- EDDHA produced maximum rooting at higher concentration than Fe-EDTA suggesting that it is more effective in terms of managing the iron supply.

Number of roots per explant: Statistical analysis showed that number of roots per explant varied significantly pertaining to the different concentrations of Fe-EDTA and Fe-EDDHA (Table 4). Fe-EDDHA produced highest number of roots (3.40) at the concentration of T_5 (200 mg l^{-1}) while in Fe-EDTA, maximum number of roots (1.82) were formed at T_1 (100 mg l^{-1}) (Fig. 5a & b). The roots formed are also morphologically different from each other. Fe-EDTA produced smaller and weak roots while Fe-EDDHA produced larger and healthy root system (Fig. 6a & b). Molassiotis *et al*., (2004) also examined that explants nourished with Fe-EDTA were chlorotic with low chlorophyll content and this fact leads to inhibition of the rooting. Zawadzka & Orlikowsk (2006) reported that addition of Fe-EDDHA in the medium not only eliminated chlorosis but also increased the amount of chlorophyll ‘a’ and ‘b’ in the red raspberry cultivars. Like the previous parameter the number of roots per explant varied with increasing the concentration of Fe-EDTA and Fe-EDDHA. However higher concentration of Fe-EDDHA had less toxic effects as compared to Fe-EDTA suggesting that Fe-EDDHA complex is more stable.

Table 4. Effect of different concentrations of Fe-EDTA and Fe-EDDHA on *In vitro* rooting of *Prunus* rootstock GF-677.

Treatments (mg l^{-1})	Rooting percentage (%)	Number of roots per explant	Root length (cm)
		Fe-EDTA	
T_0 (0.0)	0.00 f	0.00 f	0.00 f
T_1 (100)	40.00 d	1.83 d	2.57 c
T_2 (200)	30.00 e	1.41 e	1.37 d
T_3 (300)	25.00 e	1.13 e	1.00 e
		Fe-EDDHA	
T_4 (100)	60.00 c	2.19 c	2.62 c
T_5 (200)	95.00 a	3.40 a	6.88 a
T_6 (300)	70.00 b	2.55 b	3.42 b
$LSD_{0.05}$	9.076	0.3353	0.1512

Means not followed by same letter are significantly different at $p<0.05$

Fig. 5. Root formation (a) More number of roots with 200 mg l^{-1} Fe-EDDHA (T_2) (b) Fewer roots with 100 mg l^{-1} Fe-EDTA (T_1)

Root length (cm): It is clearly visible from the data that best results obtained with Fe-EDDHA showed the consistent trend as shown by the previous parameters of rooting percentage and root number. Fe-EDDHA produced longest roots (6.88 cm) at the concentration of T_5 (200 mg l^{-1}), while in Fe- EDTA maximum root length (2.57 cm) (Fig. 7a & b) at T_1 (100 mg l^{-1}) (Table 4). One probable explanation of the above outcome is that the IAA degradation is catalyzed by the photodynamic action of Fe-EDTA (Stasinopoulos & Hangarter, 1990). Since Fe-EDTA is the major light absorbing component in the tissue culture media (Stasinopoulos & Hangarter, 1990) it may be the most likely source of light induced growth reduction observed in *Arabidopsis* roots (Molassiotis *et al*., 2004).

Fig. 6. (a)Smaller and weak roots formed at 200 mg l^{-1} Fe- EDTA (T_2) (b) larger and healthy roots were observed with 200 mg l^{-1} Fe-EDDHA (T_5)

Fig. 7. Root length (a) Maximum root length observed with Fe-EDDHA at T_5 (200 mg l^{-1}) (b) Smaller root length obtained with Fe-EDTA at T_1 (100 mg l^{-1})

Antonopoulou *et al*., (2007) reported that the addition of Fe-EDTA in the culture medium greatly improved the availability of iron. Molassiotis *et al*., (2004) observed that on comparing the various iron treatments iron contents was observed to be 1.5- 2 fold higher with $FeCl_3$, 3.5 fold higher with Fe- EDDHA and 9-12 fold greater with Fe-EDTA thus at higher iron concentration Fe-EDTA began to be toxic. Moreover, Molassiotis *et al*., (2004) also suggested that this oxidative stress may lead to metabolic disfunction and ultimately in DNA damage leading possibly to inhibition of rooting. Chan & Hadar (1991) reported that Fe-EDDHA is a more stable form and suffer less hydrolysis at higher concentration than Fe-EDTA.

References

Ahmad, T. 2002. *Effect of different media composition and growth regulators on In vitro shoot proliferation and rooting of peach rootstock* GF-677. M.Sc. (Hons) Thesis, Univ. Arid. Agri., p. 2-3.

Ahmad, T., H. U. Rehman, Ch. M.S. Ahmed and M.H. Laghari. 2003. Effect of culture media and growth regulators on micropropagation of peach rootstock GF-677. *Pak. J. Bot*., 35(3): 331-338.

Ahmad, T., H.U. Rehman and M.H. Laghari. 2004. Effect of different auxins on *In vitro* rooting of the peach rootstock GF-677. *Sarhad J. Agric*., 20: 373-375.

Ainsley, P.J., G.C. Collins and M. Sedgley. 2000. Adventitious shoot regeneration from leaf explants of almond (*Prunus dulcis* Mill). *In Vitro Cell. Dev. Biol. Plant*, 36: 470-474.

Ali, A., T. Ahmad, N.A. Abbasi and I.A. Hafiz. 2009. Effect of different culture media and growth regulators on *In vitro* shoot proliferation of olive cultivar "Moraiolo". *Pak. J. Bot*., 41: 783-795.

Antonopoulou, C., K. Dimassi, I. Therios, C. Chatzissavvidis, V. Tsirakoglou and I. Papadakis. 2007. The effects of Fe-EDDHA and of ascorbic acid on *In vitro* rooting of peach rootstock GF-677 explants. *Acta Physiol. Plant*, 29: 559-561.

Assaad, F. F. and T. Awad.1981. The stability of iron chelate in calcareous soils. *Plant Physiol*., 144: 77-86.

Berthon, J.Y., R. Maldiney, B. Sotta, T. Gasper and N. Boyer. 1989. Endogenous levels of plant hormones during the course of adventitious rooting in cuttings of *Sequoiadendron gigantum* (Lindl). *In vitro*. *Biochem. Physiol. Plant*, 184: 405-412.

Bister-Miel, F., J.L. Guignard, M. Bury and C. Agier. 1985. Glutamine as an active component of casein hydrolysate: it's balancing effect on plant cells cultured in phosphorus deficient medium. *Plant Cell Rep*., 4: 161-163.

Chan, Y. and Y. Hadar. 1991. *Iron nutrition and interactions in plants*. Kluwer Academic publishers. p. 3-27.

De Groot, C.C., F.M.L. Marcelis, R.V.D. Boogaard, W.M. Kaiser and H. Lambers. 2003. Interaction of nitrogen and phosphorus nutrition in determining growth. Plant and soil. *Springer Netherland*, 248: 257-268.

George, E.F, M.A. Hall and G.J. DeKlerk. 2008. *Plant Propagation by tissue culture*. 3rd Edition. *Springer*, 1: 1-504.

Gomez, M.P. and J. Segura. 1994. Factor controlling adventitious bud induction and plant regeneration in mature *Juniperus oxycedrus* leaves cultured. *Cell. Div. Biol. Plant*, 30: 210-218.

Hangarter, R.P. and T.C. Stasinopoulos. 1991. Effect of Fe-catalyzed photooxidation of EDTA on root growth in plant culture media. *Plant Physiol*., 96: 843-847.

Igarashi, Y. and M. Yuasa. 1994. Effect of NH_4 and total nitrogen contents in the culture media on shoot regeneration from calli in saffron (*Crocus sativus* L.). *Plant Tissue Culture Letters*, 11: 61-64.

James, D.J. 1987. Cell and tissue culture technology for the genetic manipulation of temperate fruit tress. *Biotechnol. Genetic Eng Rev*., 5: 33-79.

Jaramillo, E.H. De., A. Forero, G. Cancino, A. M. Moreno, L.E. Monsalve and W. Acero. 2008. *In vitro* regeneration of three chrysanthemum (*Dendrathema grandiflora)* varieties "*via*" organogenesis and somatic embryogenesis. *Universitas Scientiarum*, 13: 118-127.

Kothari, S.L., K. Agarwal and S. Kumar. 2004. Inorganic nutrient manipulation for highly improved plant regeneration in finger millet *Eluesine coracana* (L.) *Gaertn. Cell. Div. Biol. Plant*, 40: 515-519.

Lamrioui, A.M., A. Louerguioui and A. Abousalim. 2009. Effect of the medium culture on the micro cutting of the material resulting from the adult cuttings of the wild cherry trees (*Prunus avium L*.) and of *in vitro* germination. *Eur. J. Sci. Res.*, 1: 345-352.

Leiffert, C., K.P. Murphy and P.J. Lumsden. 1995. Mineral and carbohydrate nutrition of plant cell and tissue cultures. *Crit. Rev. Plant Sci.*, 14: 83-109.

Molassiotis, A.N., K. Dimassi, I. Therios and G. Diamantidis. 2004. Fe-EDDHA promotes rooting of the rootstock GF-677 (*Prunus amygdalus x P. persica*) explants *In vitro*. *Biologia Plantarum*, 47: 141-144.

Monticelli, S., G. Puppi and G. Damiano. 2000. Effects of *In vivo* mycrorrhization on micropropagated fruit trees rootstocks. *Appl. Soil Ecol*., 15: 105-111.

Murai, M. 1994. Cytokinins biosynthesis in tRNA and cytokinins incorporation into plant RNA. *In*: Cytokinins chemistry, activity and Function. (Ed). D. W. S. Mok and M. C. Mok CRC press. pp. 87

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, 15: 473-497.

Nordstrom, A., P. Tarkowski, D. Tarkowska, R. Norbaek, C. Astot, K. Dolzel and G. Sandberg. 2004. Auxin Regulation of cytokinins biosynthesis in the *Arabidopsis thaliana*: A factor of potential importance for auxin cytokinins regulated development. www.pnas.org /cgi/doi/ 10: 1073.

Pasternak, T., P. Miskolczi, F. Ayaydin, T. Mészáros, D. Dudits and A. Feher. 2000. Exogenous auxin and cytokinin dependent activation of CDKs and cell division in leaf protoplast-derived cells of alfalfa. *Plant Growth Regul*., 32: 129-141.

Puchooa, D. 2004. *In vitro* regeneration of lychee (*Litchi chinensis* Sonn). *Afr. J. Biotech*., 3(11): 576-584.

Quoirin, M., P. Lepoivre and P. Boxus. 1977. Un premier bilan de 10 annees de recherches sur les cultures de meristemes et la multiplication *In vitro* de fruitiers ligneux. In: *Compte rendu desrecherches* 1976-1977. EJ Rapports de synthese. Station de cultures fruitieres et maraicheres, *Gembloux*, p. 93-117.

Rugini, E. and P.G. Pesce. 2006. Genetic improvement of olive. *Pomologia Croatica*, 12: 34-76.

Ruzic, D., M. Saric, R. Cerovic and I. Culafic. 2001. Changes in macronutrient content of the media and in sweet cherry Inmil GM shoots during *in vitro* culture. *J. Hort. Sci and Biotech.*, 76: 295-299.

Sajid, G.M., M.K. Ilyas and R. Anwar. 2006. Effect of diverse hormonal regimes on *in vitro* growth of grape germplasm. *Pak. J. Bot.*, 38(2): 385-391.

Shanjani, P.S. 2003. Nitrogen effects on callus induction and plant regeneration of *Juniperus excelsa*. *Int. J. Agri. Biol.*, 5: 419-422.

Shirin, F., M. Hossain, M.E. Kabir, M. Roy and S.R. Sarker. 2007. Callus induction and plant regeneration from internodal leaf explants of four potato cultivars. *World J. Agric. Sci*., 3: 1-6.

Skoog, F. and C. Miller. 1957 Chemical regulation of growth and organ formation in plant tissues cultured *In vitro*. In: *Symp. Soc. Exptl. Biol., The biological action of growth substances*, 11: 118-140.

Sriskandarajah, S., P.B. Goodwin and P. Speirs. 1994. Genetic transformation of the apple scion cultivar delicious *via Agrobacterium tumefaciens. Plant Cell Tiss. Org. Cult.*, 36: 317-329.

Stasinopoulos, T. C. and R. P. Hangarter. 1990. Preventing photochemistry in culture media by long pass light filters growth of cultured tissue. *Plant Physiol*., 93: 1465-1469.

Steel, R.G.D., J.H. Torrie and M.A. Boston. 1997. *Principles and procedures of statistics: A biometric approach*. 3rd ed., McGraue Hill Book Co. Inc NY., p.178-182.

Tang, H., Z. Ren, G. Reustle and G. Krezal. 2002. Plant regeneration from leaves of sweet and sour cherry cultivars. *Scient. Hort*., 93: 235-244.

Thrope, T.A., K. Bagh, A.J. Cutler. D. I. Datsun, D. D. McIntyre and H.J.A. Vogel. 1989. ^{14}N and ^{15}N nuclear magnetic resonance study of nitrogen metabolism in shoot forming cultures of white spruce (*Picea glauca*) buds. *Plant Physiol*., 91: 193-202.

Vander Slam, T.P.M, C.J.C.V. Toorn, C.H. Tencate, L.A.M. Dubois, D.P. Devries and H.J.D-M Don. 1994. Importance of iron chelate formula for the micropropagation of *Rosa hybrida* L. Money way. *Plant Cell Tiss. Org. Cult.*, 37:73-77.

Welander, M. 1988. Plant regeneration from leaf and stem segments of shoots raised *in vitro* from mature apple trees. *J. Plant. Physiol.*, 132: 738-744.

Williams, R.R .1995. The chemical microenvironment. In: *Automation and Environment Control in Plant Tissue Culture.* (Eds.): Aitken-Christie J, Kozai Smith Mal. Kluwer Academic Publishers, Dordrecht, p. 405-439.

Yaseen, M., T. Ahmed, N.A. Abbasi and I.A. Hafiz. 2009. Assessment of apple rootstocks M-9 and M-26 for *In vitro* rooting potential, using different carbon sources. *Pak. J. Bot*., 41(2): 769-781.

Yu, J.Q., Y. Li, Y.R. Qian and Z.J. Zhuby. 2001. Cell division and cell enlargement in fruit of *Lagenaria leucantha* as influenced pollination and plant growth substances. *Plant Growth Reg*., 33: 117-122.

Zawadzka, M. and T. Orlikowsk, 2006. The influence of Fe-EDDHA in red raspberry cultures during shoot multiplication and adventitious shoot regeneration from leaf explants, *Plant Cell Tiss. Org. Cult*., 85: 145-149.

Zhu, H.L. and M. Welander. 2000. Adventitious shoot regeneration of two dwarfing pear rootstocks and the development of a transformation protocol. *J. Hort. Sci. Biotech.*, 75 (6): 745-752.

Zhu, H.L., X.Y. Li, A. Ahlman and M. Welander. 2003. The rooting ability of the dwarfing pear rootstock BP10030 (*Pyrus communis*) was significantly increased by introduction of rol B gene. *Plant. Sci*., 165: 829-83.

(Received for publication 6 September 2009)