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EFFECT OF CULTURE MEDIA AND GROWTH REGULATORS ON MICROPROPAGATION OF PEACH ROOTSTOCK GF 677

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Abstract

The effects of Murashige & Skoog (1962) and Anderson (1984) media with different BA (benzyladenine) concentrations (0.3, 0.6, 0.9 mg l^{-1}) on the *in vitro* shoot proliferation of peach rootstock GF 677 was investigated. Shoot proliferation, elongation and growth on MS medium was the best whereas on AND medium the shoots were chlorotic, small and vitrified. BAP 0.6 mg l^{-1} produced higher number of shoots having > 2 cm length. Higher levels of BAP (0.9 mg l^{-1}) induced callus formation and shoot apical necrosis. The best root system was developed on half strength MS media supplemented with 3 mg l^{-1} indolebutyric acid (IBA). Higher levels of IBA (4.0 mg l^{-1}) induced callus and inhibited normal root development.

Introduction

Historically, seedlings were used as rootstocks and grafting was exploited as asexual propagation method to multiply fruit tree clones. Little concern was attributed to the rootstock characteristics other than compatibility with the scion. Today rootstocks are also used to meet specific cultural needs. Although for fruit tree species seed-propagated rootstocks are still used, there are many rootstocks propagated asexually as clones. A peach rootstock GF 677, an interspecific hybrid (Peach x Almond) is propagated asexually as clone. It is specially used on alkaline soils because of resistance to lime induced iron chlorosis (El Gharbi & Jraidi, 1994). It is highly vigorous and counteracts low soil fertility (Carrera *et al.*, 1998). However, it is difficult to be multiplied on mass scale through cuttings because of very low rooting percentage (Ammer, 1999). Tissue culture methods are available for many *Prunus* species and cultivars (Eldeen *et al.*, 1998; Perez *et al.*, 1999; Ružic *et al.*, 2000). Differences exist among the various genotypes. This study was aimed to compare MS and AND media, to explore BAP concentration best for shoot proliferation and also IBA concentration best for *in vitro* rooting of micro-cuttings.

Materials and Methods

The experiment was conducted at the *in vitro* laboratory of Plant Genetic Resources Institute, National Agricultural Research Center, Islamabad, Pakistan. Shoot tips of GF 677 from 10 years old hedge (row plantation) were collected during spring 2001 from fruit crop germplasm block of Horticultural Sciences Institute, NARC, Islamabad.

Shoot tips 3-4 cm long were taken and primary cleaning was done following Khattak *et al.*, (1990). These tips were then placed in different concentrations of Clorox (Abudawood & Partners, Saudi Arabia) giving 1.25, 1.00, 0.75, 0.5, 0.25% w/v sodium hypochlorite (NaOCl), with 1 drop of Tween-80 (Sigma^R USA) per 50 ml of solution.

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Explants were agitated in the solution for 10 minutes, the sterilizing solution was decanted, the tips were transferred into five rinses (5min each) of sterile distilled water and explants were prepared following Norton (1986).

Media used for culture establishment: Explants prepared from the above treatment were cultured individually in 25 ml test tube containing 8 ml of MS (Murashige & Skoog, 1962) medium (MS macro, micro elements, vitamins, 150 mg l^{-1} ascorbic acid, 100 mg l^{-1} citric acid, 30 g l^{-1} sucrose and 6.5 g l^{-1} agar). The pH of the culture medium was adjusted to 6 before autoclaving at 121°C, for 15 minutes. Cultures were always incubated at 25 ± 1°C under 16-h light (2,000 lux) with white fluorescent tubes. Observations on necrosis, infection and survival percentage of cultures were recorded 4 weeks after the inoculation.

Media used for shoot proliferation study: For the shoot proliferation study, established shoot cultures were transferred individually to each 25 ml test tube containing 8 ml of two shoot proliferation media *viz.*, MS and AND media (Anderson, 1984). Both the media were supplemented with MS vitamins, 30 g l^{-1} sucrose and 6.5 g l^{-1} agar. The pH of both the culture media was adjusted to 6 before autoclaving. Varying levels of BAP compared are mentioned in Table 1.

Table 1. Media composition compared for shoot proliferation.

	M_1 MS (MS macro, micro elements & MS vit) medium			M_2 AND (AND macro, micro elements & MS vit) medium		
Treatments	**BAP mg l^{-1}**	**GA_3 mg l^{-1}**	**NAA mg l^{-1}**	**BAP mg l^{-1}**	**GA_3 mg l^{-1}**	**NAA mg l^{-1}**
T_1	0.3	0.5	0.01	0.3	0.5	0.01
T_2	0.6	0.5	0.01	0.6	0.5	0.01
T_3	0.9	0.5	0.01	0.9	0.5	0.01

The experiment was a two factor factorial (Media x BAP) randomized in CRD (completely randomized design) with three replications per treatment and six shoots per replication. Data were recorded on shoot proliferation rate, total number of shoots per proliferating explant and number of usable shoots (shoots more than 2.0 cm in length) per proliferating explant after 4 weeks.

Media used for rooting: For rooting, proliferated uniform shoots were transferred individually to each 25 ml test tube containing about 8 ml of rooting medium consisting of MS half macro, micro elements and vitamins supplemented with 15 g l^{-1} sucrose and 6.5 g l^{-1} agar. The pH of the rooting medium was adjusted to 6 before autoclaving. Varying levels of IBA compared are mentioned in Table 2.

Table 2. Media composition for rooting.

	MS (half macro, micro elements and vitamins) medium
Treatments	**IBA mg l^{-1}**
T_0	0
T_1	1
T_2	2
T_3	3
T_4	4

The experiment was completely randomized design (CRD) consisting of three replications per treatment and five shoots per replication. Data were recorded on the number of rooted explants and the length and the number of roots per rooted explant after four weeks.

Results and Discussion

In vitro culture establishment

Effect of NaOCl on establishment and disinfestation of explant

The effect of different treatments on disinfestation of peach shoot tip explants after 4 weeks is presented in Table 3. It is evident from the data that 0.25% NaOC1 had significantly increased the survival percentage (55) of the cultured shoot tips. At 1% NaOC1, minimum survival percentage (5) was observed.

Higher concentration increased necrosis, which indicates that along with disinfection, young shoot tips might have been damaged, thus resulting in death of shoot tips. It was observed that peach shoot tips were sensitive to NaOC1 and high concentration could damage the tissues. Table 3 also revealed that contamination was highest (40%) when NaOCl at 0.25% concentration was used, followed by 35, 30, 20 and 0% contamination at 0.5, 0.75, 1.0 and 1.25% solution, respectively. Thus it can be concluded that although increased concentration of NaOCl can effectively control contamination however, its higher concentration badly damages the explants. For the establishment of *in vitro* culture of any plant species, it is of prime importance to find out the safe sterilization agent that can remove the fungus and bacteria from explant tissue (Fiorino & Loreti, 1987). Skirvin (1984) has reviewed different schemes for sterilization of explants from stone fruits showing varying degree of contamination. However, treatment with a 0.5-0.75% w/v NaOCl depending upon the fruit crop, has been found to be very effective in controlling high contamination rates (Hu & Wang, 1983). The same result was achieved in the present study with the application of NaOCl at 0.25% (w/v), where minimum necrosis (5%) and maximum survival (55%) was observed.

Table 3. Effect of different levels of NaOCl on percentage of necrosis, infection and survival of cultured shoot tip explants of peach after 4 weeks.

NaOCl Solution (%w/v)	Necrosis (%)*	Infection (%)*			Survival (%)*
		Bacterial	Fungal	Total	
1.25	100	0	0	0	0
1	75	5	15	20	5
0.75	50	5	25	30	20
0.5	35	10	25	35	30
0.25	5	5	35	40	55

*20 explants / treatment

***In vitro* shoot proliferation**

Effect of different culture media (MS & AND) and BAP concentrations on *In vitro* shoot proliferation rate

Non-significant differences were noted between culture media (MS & AND) in the rate of shoot proliferation (Table 4). After four weeks of culture, 100% of the shoot tip explants proliferated. However, morphologically proliferated cultures were better on MS medium; whereas the proliferated shoots were weak and yellow with some symptoms of vitrification on AND medium. With MS medium, shoots were elongated, thickened and intensively green in colour. In general, the best response was observed with MS medium for shoot proliferation rate. This might be due to the higher contents of nitrogen in this medium. These results are in accordance with Ruzic *et al.*, (2000).

Table 4. Effect of different culture media (MS & AND) and BAP concentrations on *in vitro* shoot proliferation rate in peach rootstock (GF 677).

Treatments BAP (mg l^{-1})		*Shoot proliferation rate (%)		
		**MS	***AND	Mean
T_1	0.3	100 a	100 a	100 A
T_2	0.6	100 a	100 a	100 A
T_3	0.9	100 a	100 a	100 A
Mean		100 A	100 A	
Statistical significance		Media (MS & AND) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.00	Interaction (Media x Treatments) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.00	Treatments (T_1x T_2 x T_3) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.00

*% of explants producing >1 shoot
**MS = MS medium (Murashige & Skoog, 1962)
***AND = AND medium (Anderson, 1984)
Means followed by the same letter are not statistically different.

Effect of different culture media (MS & AND) and BAP concentrations on number of shoots per proliferated explant

Significant differences were observed between culture media (MS & AND) for the number of shoots per proliferated explant (Table 5). The number of shoots per proliferated explant was significantly higher (3.75) at $p<0.05$ on MS medium as compared to AND medium resulting 2.93 shoots per proliferated explant. The higher concentration of nitrogen (1471 mg l^{-1}) and potassium (781 mg l^{-1}) in MS medium might be linked to the higher number of shoots per culture. Crawford (1995) reported that nitrogen may function as a signal molecule of plant growth *via* increased gene expression for enzyme responsible for the uptake and utilization of nitrate. Ludden & Carlson, (1980) found that growth on a poor nitrogen source is not sufficient, to cause the induction of enzymes (nitrate reductase and nitrite reductase).

Statistical analysis showed that the interaction between the culture media (MS & AND) and BAP was significant at $p<0.05$ for number of shoots per proliferated explant (Table 5). The results showed that MS + T_2 (0.6 mg l^{-1} BAP) produced highest number of shoots (6.22) per explant, followed by AND + T_2 (0.6 mg l^{-1} BAP) which produced 3.83 shoots per explant.

From these results, it is clear that there is strong interaction between the macroelements and BAP present in the culture media. McCown & Sellmer, (1987) reported that the effect of growth regulators can be strongly modified by the medium on which the cultures are grown. The interaction indicated that the most appropriate mineral salt medium was MS (high nitrogen and potassium).

High BAP concentration significantly reduced shoot multiplication in both MS and AND media (Table 5). It was observed that higher levels of BAP induced callus formation and shoot apical necrosis. It started as wilting or rotting of the apex and proceeded basipetally, causing the death of shoots. Apical necrosis might be interfering with apical dominance and development of axillary shoots (Muleo *et al.*, 1995). Skirvin (1984) reported that callus formation is antagonistic to shoot proliferation.

Table 5. Effect of different culture media (MS & AND) and BAP concentrations on number of shoots per proliferated explant in peach rootstock (GF 677).

Treatments BAP (mg l^{-1})		Mean number of shoots per proliferated explant *MS	**AND	Mean
T_1	0.3	2.05 d	1.05 e	1.55 C
T_2	0.6	6.22 a	3.83 b	5.02 A
T_3	0.9	2.99 c	1.99 d	2.49 B
Mean		3.75 A	2.93 B	
Statistical significance		Media (MS & AND) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.17	Interaction (Media x Treatments) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.29	Treatments (T_1 x T_2 x T_3) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.21

*MS = MS medium (Murashige & Skoog, 1962)

**AND = AND medium (Anderson, 1984)

Means followed by the same letter are not statistically different.

Effect of different culture media (MS & AND) and BAP concentrations on number of shoots more than 2.0 cm in length per proliferated explant

Significant differences were observed between culture media (MS & AND) for the number of shoots more than 2.0 cm in length per proliferated explant (Table 6). The number of usable shoots (shoots more than 2.0 cm in length) per proliferated explant were significantly higher (2.23) at $p<0.05$ on MS medium as compared to AND medium resulting 0.77 shoots more than 2.0 cm in length per proliferated explant. The shoots produced on the MS medium were healthy green and more uniform, whereas those on the AND medium were chlorotic, small and claviform. The improvement of shoot growth and morphology attained during preceding proliferation stage on MS medium were probably effective in preparing the shoots more than 2.0 cm in length, which nevertheless proved more satisfactory on AND medium. The depressed growth on AND medium is probably due to the high phosphate (85.0 mg l^{-1}) level. Dobberstein & Staba, (1969) showed that higher levels of phosphate increased the indole alkaloids in the medium of *Ipomoea* suspension cultures which resulted in decreased growth.

Iron chlorosis on AND medium is probably due to deleterious effect of excessive EDTA (74.5 mg l^{-1}) in the medium which may displace iron from chelate complex forming corresponding heavy metal chelates (Teasdale, 1987). The other plausible reason

may be that high phosphorus and low potassium in the AND medium is antagonistic to absorption of iron (Jolley *et al.*, 1988).

Statistical analysis showed that the interaction between the culture media (MS & AND) and BAP was significant at $p<0.05$ for number of shoots more than 2.0 cm in length per proliferated explant (Table 6). Maximum number of shoots (3.94), more than 2.0 cm in length per proliferated explant were produced on MS + T_2 (0.6 mg l^{-1} BAP) followed by AND + T_2 (0.6 mg l^{-1} BAP) and MS + T_3 (0.9 mg l^{-1} BAP) which produced 1.72 and 1.66 shoots more than 2.0 cm in length per proliferated explant, respectively. Moreover, trend set forth by the previous character has been maintained here i.e., MS + T_2 (0.6 mg l^{-1} BAP) induced more number of shoots more than 2.0 cm in length per proliferated explant.

It may be inferred from these results that there is a synergism between the number of shoot per proliferated explant and number of shoots more than 2.0 cm in length per proliferated explant i.e., greater the shoot multiplication, greater the number of shoots more than 2.0 cm in length. These results contradicted the findings of other researchers (Lloyd & McCown, 1980; Maarri *et al.*, 1986), but our results could be at least partly due to the better growth that the shoots had shown during the proceeding proliferation on MS + T_2 (0.6 mg l^{-1} BAP).

High BAP concentration inhibited shoot elongation and stimulated callus formation in both MS and AND media (Table 6). Hu & Wang, (1983) reported that cytokinins, especially BAP stimulated axillary bud development but at higher concentration shoot elongation was suppressed.

Table 6. Effect of different culture media (MS & AND) and BAP concentrations on number of shoots more than 2.0 cm in length per proliferated explant in peach rootstock (GF 677)

Treatments BAP (mg l^{-1})		Mean number of shoots more than 2.0cm in length per proliferated explant. *MS	**AND	Mean
T_1	0.3	1.10 c	0.00 e	0.55 C
T_2	0.6	3.94 a	1.72 b	2.83 A
T_3	0.9	1.66 b	0.61 d	1.13 B
Mean		2.23 A	0.77 B	
Statistical significance		Media (MS & AND) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.15	Interaction (Media x Treatments) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.27	Treatments (T_1 x T_2 x T_3) $p<0.05$ $LSD_{5\%}$ (17 d.f.) 0.19

*MS = MS medium (Murashige & Skoog, 1962)

**AND = AND medium (Anderson, 1984)

Means followed by the same letter are not statistically different.

In vitro rooting

Effect of IBA concentrations on *In vitro* rooting

Significant differences were noted between the treatments at $p<0.05$ for the rooting rate (Table 7). The results showed that T_3 (3.00 mg l^{-1} IBA) produced the higher percentage of rooted shoots (73.33 %), followed by T_2 (2.00 mg l^{-1} IBA) and T_4 (4.00 mg l^{-1} IBA) which produced 46.67 and 33.33% rooted shoots, respectively.

Table 7. Effect of IBA on *in vitro* rooting ability in peach rootstock (GF 677).

Treatments IBA (mg l^{-1})	Rooting rate (%)	Mean number of roots per rooted explant	Mean number of roots more than 1.5 cm in length per rooted explant
T_0 0.00	0.00 d	0.00 e	0.00 d
T_1 1.00	20.00 c	1.20 d	0.00 d
T_2 2.00	46.67 b	3.13 b	1.26 b
T_3 3.00	73.33 a	5.86 a	3.13 a
T_4 4.00	33.33 bc	2.20 c	0.80 c
Statistical significance			
Treatments (T_0 x T_1 x T_2 x T_3 x T_4)	p<0.05 $LSD_{5\%}$ (14 d.f.) 0.22	p<0.05 $LSD_{5\%}$ (14 d.f.) 0.35	p<0.05 $LSD_{5\%}$ (14 d.f.) 0.35

Means followed by the same letter are not statistically different.

From these results it is clear, that for root formation auxin seems to be indispensable, as rooting did not occur in the absence of IBA (Table 7). Hu & Wang (1983) reported that auxin exerted the primary control over root formation.

Increasing levels of IBA had a tendency to increase rooting of shoots (Table 7). However, at higher concentration (4.00 mg l^{-1} IBA) it was inhibited. T_4 (4.00 mg l^{-1} IBA) showed non-significant difference with T_1 (1.00 mg l^{-1} IBA) and T_2 (2.00 mg l^{-1} IBA). This might be due to the inhibitory effect of IBA. It has been reported that IBA enhances rooting at low concentrations but inhibits it at higher concentrations (Zimmerman, 1984). A lower doze T_3 (3.00 mg l^{-1} IBA) proved better than higher regime (4.00 mg l^{-1} IBA). Results are also supported by Werner & Boe (1980).

Significant differences were noted between the treatments at p<0.05 for the number of roots per rooted explant (Table 7). The result showed that T_3 (3.00 mg l^{-1} IBA) produced maximum number of roots (5.86) per rooted explant followed by T_2 (2.00 mg l^{-1} IBA) which produced 3.13 roots per rooted explant. These results showed almost similar pattern as that of previous results (Table 7).

From these results it is clear, that the number of roots per rooted shoots increased with increasing IBA concentration (Table 7) but at higher concentration it decreased. It was observed that higher levels of IBA induced callus. Hu & Wang (1983) reported that when the auxin concentration is too high, callus would form as the shoot base inhibiting normal root development.

Significant differences were noted between the treatments at p<0.05 for the number of roots more than 1.5 cm in length per rooted explant (Table 7). Maximum number of roots more than 1.5 cm in length per rooted explant (3.13) were produced on T_3 (3.00 mg l^{-1} IBA) followed by T_2 (2.00 mg l^{-1} IBA) which produced 1.26 roots more than 1.5 cm in length per rooted explant. These results indicate a positive correlation between number of roots and number of roots more than 1.5 cm in length. The best results were obtained with 3.00 mg l^{-1} IBA (T_3) without callus interphase. However, higher concentration (4.00 mg l^{-1} IBA, T_4) induced callus with the roots. The results are supported by the findings of Hu & Wang, (1983) who indicated that root elongation phase was very sensitive to auxin concentrations and was inhibited by higher concentrations.

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