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Effect of Different Growth Regulators and Source of Carbohydrates on *in* and *ex vitro* Rooting of Iranian Myrtle

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Abstract: Nodal segments of wild Iranian myrtle was used to study the effects of two carbohydrate sources on shoot proliferation and to determine residual effect of different growth regulators and carbohydrate sources on *in* and *ex vitro* rooting. Half strength Murashige and Skoog (MS) medium was used for both shoot proliferation and rooting. The highest shoot proliferation (27.3 shoots/explant) observed in medium containing 2 mg L^{-1} b-benzylamino purine (BA) with 0.2 mg L^{-1} Indole-3-acetic acid (IAA) (M7) or 0.2 mg L^{-1} α-naphthaleneacetic acid (NAA) (M5). There were no significant differences between two sources of carbohydrates on shoot proliferation at all growth regulators levels. Maximum root numbers/shoot (3.8) observed on shoots originating from media containing 2 mg L^{-1} BA and 0.2 mg L^{-1} NAA on rooting media containing 0.5 mg L^{-1} IAA. The rate of rooting increased from zero% in non treated shoots to 91.7% when the basal end of shoots were dipped in a solution of 1.5 mg L^{-1} IAA and 0.31 mg L^{-1} Indole-3-butyric acid (IBA) for 24 h before culturing in soil mixture. A procedure for *ex vitro* rooting was proposed in order to decrease the micropropagation cost.

Key words: Carbohydrate, residual effect, α-naphthaleneacetic acid, b-benzylamino purine, Indole-3-acetic acid

Introduction

Myrtle (*myrtus communis* L.) belonging to the Myrtaceae family is an evergreen aromatic shrub, that wildly grows in Fars province of Iran. This species is valued for ornamental purposes. The medicinal uses of myrtle extract have been reported as antimicrobial (Salih and Nodir, 1984), stimulant, astringent, antiseptic and bactericide (Scarpa *et al*., 2000). Today, in addition to the increased popular uses of medicinal plants instead of industrial pharmaceutical products, the use of medicinal plants as materials for the extraction of active pharmacological agent or as a precursors for chemo-pharmaceutical hemi-synthesis has been increased (Magherini, 1988).

In order to increase the cultivation of *M. communis* L., the first step is the production of large quantities of high quality genetically homogeneous plant material that can be propagated at low prices.

Micropropagation of myrtle using various explants sources such as apical meristem (Parra and Amo-Marco, 1998), nodal segments (Nobre, 1994; Parra and Amo-Marco, 1996), shoot tip culture (Khosh-Khui *et al*., 1984), axillary shoots (Ruffoni *et al*., 2003) and also the effect of different growth regulators on *in vitro* rooting has been successfully investigated (Hatzilazarou, *et al*., 2003). From a commercial standpoint, the induction of roots *in vitro* is an expensive labor-consuming process, which may account for 35-75% total cost of plant propagated *in vitro* (Debergh and Maene, 1981). The residual composition of proliferation media (carbohydrates and plant growth regulators) affects the shoots rooting and their survival in the soil (Nobre, 1994; Hazarika, 2003). Wainwright and Scrace (1989) found that maximum values for shoot height and fresh and dry weight of *Potentilla fruticosa* and *Ficus lyrata* were obtained *ex vitro* when preconditioned with 2 or 4% sucrose. Plantlets establishment declined when sucrose was not used.

The aim of this research was, to study the effect of tissue culture-grade sucrose and table sugar on nodal segment proliferation of wild Iranian myrtle and also to study *in* and *ex vitro* rooting of *in vitro* derived shoots for decreasing the cost of myrtle micropropagation.

Materials and Methods

Newly growing shoots (5 to 10 cm) were collected in May 2003 and 2004 from wild myrtle plants in Fassa region near Shiraz in Fars province (Fig. 1a). Shoots were washed thoroughly under running tap water for 1 h, then agitated in a solution of 2 g L^{-1} benomyl for 30 min. For removal of

Fig. 1: Developmental stages of micropropagated myrtle plantes. (a) Wild myrtle in which the explants were collected. (b) shoot proliferation after 3 months of culture. (c) rooting of shoots in MS/2 with 1 mg L^{-1} IAA. (d) six weeks old rooted plantlets in soil. (e) sixteen months old micropropagted plants in the field

Table 1: Shoot proliferation media supplemented with growth regulators

Media	Plant growth regulator
M1	0.0 mg L^{-1} BA
M2	1.5 mg L^{-1} BA
M3	2.0 mg L^{-1} BA
M4	1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA
M5	2.0 mg L^{-1} BA + 0.2 mg L^{-1} NAA
M6	1.5 mg L^{-1} BA + 0.2 mg L^{-1} IAA
M7	2.0 mg L^{-1} BA + 0.2 mg L^{-1} IAA

phenolic compounds the shoots were immersed in an antioxidant solution (75 mg L^{-1} citric acid + 75 mg L^{-1} ascorbic acid) for 45 min. Shoots were sterilized in 15% Golrang (a commercial bleach containing 5.25% sodium hypochlorite plus a few drop of Rica (a commercial detergent) for 15 min and then rinsed 3 times with sterilized distilled water.

Nodal segments (0.5 to 1 cm) were cultured on shoot proliferation medium. On the basis of our previous work half strength basal salt Murashige and Skoog (1962) medium plus (mg L^{-1}): nicotinic acid, 0.5; pyridoxine HCl, 0.5; thiamine, 0.4; glycine, 2.0; inositol 100 (Khosh-Khui *et al*., 1984), with different concentration of plant growth regulators shows in Table 1 was used.

For evaluating the effect of sucrose and table sugar on shoot proliferation, the proliferation media were supplemented with 30 g L^{-1} sucrose (MERCK, LGaA 64271 Darmstadt, Germany) and white table sugar (available in market). The culture was carried out in 250 mL Erlenmeyer containing 30 mL of growth medium each in which 3 nodes were transplanted. They were maintained at 25±1°C under 16 h cool-white fluerescent tubes with 2500 lux light intensity. The number and the length of shoot from each primary node was recorded 8 weeks after culture.

For studying the effect of different plant growth regulators residual on rooting, the shoots (2 cm long) derived from media M3 (2 mg L^{-1} BA), M5(2 mg L^{-1} BA + 0.2 mg L^{-1} NAA) and M7 (2 mg L^{-1} BA + 0.2 mg L^{-1} IAA) were cultured separately in culture tubes (15 cm length and 2.5 cm diameter) containing 15 mL half strength MS medium supplemented with 0, 0.5 and 1 mg L^{-1} IAA.

For evaluating the residual effect of carbohydrates, on *in vitro* rooting, the shoots derived from media supplemented with white table sugar or sucrose were cultured on half strength MS basal medium supplemented with 0, 0.25, 0.5 and 1 mg L^{-1} IAA.

All media were solidified with 0.7% agar-agar (MERCK, LGaA 64271 Darmstadt, Germany), pH adjusted to 5.75 ± 0.05 and autoclaved for 15 min at 120°C and 1.5 atm. pressure.

For studying *ex vitro* rooting, (based on a preliminary work using IAA, NAA and IBA, unpublished data), 128 shoots (2 cm long) were treated for 24 h in various auxin solutions as fallows: 4.5 mg L^{-1} IAA, 3 mg L^{-1} IAA + 0.15 mg L^{-1} IBA, 1.5 mg L^{-1} IAA + 0.31 mg L^{-1} IBA and 0.6 mg L^{-1} IBA (32 shoots/treatment) and then, they were cultured on non-sterilized mixture of soil: sand and peat moss (1:1) and covered with plastic bags.

All the cultures were maintained at 24±1°C under 16 h photoperiod, provided by cool-white fluorescent tubes with 1500 lux light intensity. For acclimatization, the *in vitro* rooted shoots were washed to remove agar and planted in a soil mixture containing equal parts of peat and vermiculite (by volume) and covered with transparent plastic bags which were gradually removed during the first 2 weeks.

For acclimatization of *ex vitro* rooted shoots, the plastic bags were removed during 4 weeks and after 6 weeks, 16 shoots of each treatment were removed from the soil to record the root number and length and the rest of the shoots (16 shoots) were maintained in the same condition for further data recording.

All experiments were carried out in a Complete Randomized Design (CRD) with different replications and number of plants per each treatment specified in the bottom of each Table. Data were analyzed using SPSS statistical software (SPSS Ine. Chicago, UAS), the means were compared by Duncan's multiple range tests.

Results

Shoot proliferation began as early as 1st week after placing nodal segments on various media with different concentrations of growth regulators and carbohydrates with no callus formation. The addition of plant growth regulators in the culture media increased shoot proliferation in comparison to the control (Table 2). Average shoot number/explant increased significantly by addition of 1.5 and 2 mg L^{-1} BA without auxin in medium. Adding 0.2 mg L^{-1} auxin (IAA or NAA) with BA, significantly promote shoot proliferation. After 8 weeks, the highest shoots proliferation observed in M7 medium

Table 2: Effect of plant growth regulators and source of carbohydrate on shoot number and length of wild myrtle.†

	Carbohydrate (30 g L^{-1})		
	Table sugar	Sucrose	Media
Media	Average shoot no. per explant	Average shoot no. per explant	Mean††
Ml (control)	01.3e	01.58e	01.5E
M2	10.2d	10.42d	10.3D
M3	12.7c	13.25c	12.9C
M4	18.9h	20.08b	19.50B
M5	26.2a	28.42a	27.3A
M6	21.2b	20.50b	20.8B
M7	26.5a	28.16a	27.3A
Mean	16.7A	17.48A	
Media	Average shoot length per explant (cm)	Average shoot length per explant (cm)	
Ml	3.6a	3.7a	3.6A
M2	2.6bcd	2.2bcde	2.2B
M3	2.4bc	2.2bcde	2.3B
M4	2.2bcde	2.2bcde	2.2B
M5	l.8ef	1.7f	1.8B
M6	1.9def	2.5b	2.2B
M7	2.3bcd	2.1cde	2.2B
Mean	2.4A	2.4A	

† Results based on 4 replications of 3 explants per each treatment, †† Means in each column and row with the same small letters and mean in each column and row with same capital letters are not significant at 5% level of probability using Duncan's multiple range test. Ml= 0.0 mg L^{-1} BA, M2=1.5 mg L^{-1} BA, M3= 2.0 mg L^{-1} BA, M4= 1.5 mg L^{-1} BA + 0.2 mg L^{-1} NAA, M5 = 2.0 mg L^{-1} BA + 0.2 mg L^{-1} NAA, M6 = 1.5 mg L^{-1} BA + 0.2 mg L^{-1} IAA, M7= 2.0 mg L^{-1} BA + 0.2 mg L^{-1} IAA

Table 3: The residual effect of growth regulators used in proliferation media on rooting of myrtle shoots†

	IAA used in rooting media mg L^{-1}			
	0	0.5	1.0	
Proliferation media with various growth regulators	Average root No. per explant			Mean††
M3	0.3d	1.7c	2.4bc	1.5B
M5	0.8d	3.8a	3.8a	2.8A
M7	0.9d	2.9b	3.7a	2.5A
Mean	0.7C	2.8B	3.2A	
	Average root length per explant (cm)			Mean
M3	1.0b	2.2a	2.4a	1.9A
M5	1.4b	2.5a	2.4a	2.0A
M7	1.4b	2.5a	2.5a	2.1A
Mean	1.3B	2.3A	2.4A	

†Results based on 16 replicates of one shoot per each treatment, †† Means in each column and row with the same small letters and mean in each column and row with same capital letters are not significant at 5% level of probability using Duncan's multiple range test

Table 4: Effect of different concentrations of IAA on rooting of myrtle shoot regenerated from M7 containing white table sugar†

IAA mg L^{-1}	Rooting (%)	Avarege shoot No. per explant	Average length of root per shoot (cm)	Rooted plants survival in soil (%)
0.0	31.3	1.0c††	1.6a	40
0.25	62.3	3.0b	1.8a	60
0.50	87.5	4.6a	1.8a	85.7
1.00	87.5	3.7b	1.0b	78.5

† Results based on 16 shoots per each treatment, †† Means in each column with the similar letters are not significant at 5% level of probability using Duncan's multiple range test

Table 5: Effects of different concentrations of growth regulators on rooting of myrtle shoot *ex vitro* †

Growth regulators mg L^{-1}	Rooting %	Average root No./explant	Average length of root per shoot (cm)
IAA 4.5	50	2.3b††	1.9a
IAA3+IBA 0.15	75	2.8a	1.5b
IAA 1.5+IBA 0.31	91.7	3.3a	1.2b
IBA 0.6	75	3.5a	1.2b

† Results based on 16 shoots per treatment, †† Means in each column with the similar letters are not significant at 5% level of probability using Duncan' s multiple range test

containing 2 mg L^{-1} BA + 0.2 mg L^{-1} IAA (27.3 shoots/explant) and M5 medium containing 2 mg L^{-1} BA + 0.2 mg L^{-1} NAA (27.3 shoots/explant). After three months, the shoots produced auxiliary shoots so that the total number of shoots/explants was 150 (Fig. 1b).

Effect of interactions between plant growth regulators and two sources of carbohydrate on the rate of proliferation showed that in all levels of growth regulators there was no significant difference between the two sources of sugar (Table 2). Not considering the effect of carbohydrate, increasing the BA concentration with or without auxin (IAA or NAA) decreased average shoot length from 3.6 cm in M1 medium (control) to 1.8 cm in M5 medium containing 2 mg L^{-1} BA + 0.2 mg L^{-1} NAA. The carbohydrate sources had the same effect on average shoot length in all levels of growth regulators except in M6 medium with 1.5 mg L^{-1} BA and 0.2 mg L^{-1} IAA where the average length of shoots was 2.5 cm in media containing sucrose vs. 1.9 in media containing white table sugar.

Effects of interactions between the residual effect of BA with or without auxin (NAA or IAA) and different concentrations of IAA on rooting revealed that, the shoots originating from M5 (2 mg L^{-1} BA + 0.2 mg L^{-1} NAA) and M7 (2 mg L^{-1} BA + 0.2 mg L^{-1} IAA) media induced better roots than the shoot originating from M3 (2 mg L^{-1} BA) medium. The highest root number was obtained for shoot originating from M5 medium cultured on rooting media containing 0.5 mg L^{-1} IAA (3.8 roots/shoot) followed by shoots originating from M7 containing 2 mg L^{-1} BA + 0.2 mg L^{-1} IAA (2.9 roots/shoot). Increasing IAA from 0.5 to 1 mg L^{-1} ameliorate rooting of shoots originated from M7 (3.7 root/shoot) (Fig. 1c).

The residual effect of plant growth regulators had no significant effect on average root length but addition of 0.5 or 1 mg L^{-1} IAA to rooting media significantly improved average root length (Table 3).

The results of rooting shoots from M7 supplemented with white table sugar in different IAA concentrations were shown in Table 4. The highest rooting percentage was observed in media containing 0.5 and 1 mg L^{-1} IAA (87.5%). Maximum average root numbers/shoot was obtained in media containing 0.5 mg L^{-1} IAA (4.6 roots/shoot) which is significantly higher than control (1.0 root/shoot) and 0.25 mg L^{-1} IAA (3 roots/shoot) and 1 mg L^{-1} IAA (3.7 roots/shoot), also the plantlets rooted in medium containing 0.5 mg L^{-1} IAA survived better in soil than the other treatments (85.7%). The same results were observed in shoots originated from media supplemented with sucrose (data not shown). Increasing IAA to 1 mg L^{-1} in rooting media reduced root length to 0.98 vs. 1.6 cm for the control.

In a preliminary experiment, the rooting percentage was zero when the shoots were cultured in soil mixture without any treatment. The effect of growth regulators treatment on *ex vitro* rooting

revealed that, using only IBA or combination of IBA and IAA increased rooting percentage and average root number/shoot (Table 5). The highest rooting percentage was observed in shoots treated with 1.5 mg L^{-1} IAA + 0.3 mg L^{-1} IBA (91.7%). This treatment also produced 3.3 roots/shoot which was significantly higher that using only 4.5 mg L^{-1} IAA (2.2 roots/shoot).

Discussion

In the present investigation high shoot proliferation of wild Iranian myrtle was possible in the presence of half strength MS salts, 2 mg L^{-1} BA and 0.2 mg L^{-1} auxin (NAA or IAA) and 30 g L^{-1} sugar. Incidence of hyperhydricity that was reported by Parra and Amo-Marco (1998) in Mediterranean myrtle have never been observed in our experiments. This may be result of explant sources of different cultivars.

Tissue and organ culture requires a carbohydrate supply in order to satisfy energy demands. Among the many available carbon sources, sucrose has been the major one (Peterson *et al*., 1999; Fuentes *et al*., 2000). There were no significant differences in induction of shoot number per node or root number per shoot on media containing white table sugar or tissue culture grade-sucrose. Addition of auxin (NAA or IAA) and BA source not only is necessary to promote *in vitro* shoot proliferation (Khush-khui *et al*., 1984; Nobre 1994; Scarpa *et al*., 2000), but also their residual effect promote number of roots/shoot in comparison to BA only that inhibits root proliferation.

Most reports of adventitious root induction of woody species have involved treatment with exogenous auxin such as IBA, NAA and IAA (Torres, 1988; George, 1996 cited in Anisly *et al*., 2001). It has also been proposed that induction occurs within a few hours of auxin application and that prolonged exposure may not be necessary (Collet, 1988). Our case was the same, the rate of rooting was zero% in *ex-vitro* without any treatment, therefore, the roots were obtained by dipping the basal end of the shoot in au auxin solution for 24 h (Table 5). The highest rooting rate (% of responsive shoots and number of roots/shoot) were obtained using a solution of 1.5 mg L^{-1} IAA + 0.31 mg L^{-1} IBA. Martin (2003) also facilitated the survival of 75% of shoots in soil after dipping the basal end of shoots of *Rotula aquatica* Lour in 0.5 mg L^{-1} NAA for 25 days. Scarpa *et al*. (2000) mentioned that roots were obtained by transplanting myrtle plantlets which were directly cultured in the soil showed higher percent of survival in the field than those rooted under *in vitro* conditions. Laber cost can be dropped considerably with *ex vitro* rooting.

Generally, it appears that the best treatment for *in vitro* propagation of myrtle is a combination of half strength MS containing low price white table sugar instead of expensive tissue culture-grade sucrose supplemented with 2 mg L^{-1} BA + 0.2 mg L^{-1} NAA for proliferation and then direct transplantation of shoots to the soil mixture after treatment with IBA (0.6 mg L^{-1}) or combination of IBA (0.31 mg L^{-1}) and IAA (1.5 mg L^{-1}) reduces the time of acclimatization and labor cost. Young myrtle plantlets after above treatments were rooted in soil (Fig. 1d). Transplanted plantlets were then established in the field successfully (Fig. 1e).

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