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Effect of plant growth regulators and basal media on *in vitro* shoot proliferation and rooting of *Myrtus communis* L.

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Abstract

The influence of macronutrients and growth regulators on *in vitro* shoot proliferation and rooting of an East Spanish population of *Myrtus communis* L. were studied. Preincubation of field material on a medium without mineral salts prevented the browning from phenolic exudates. For multiplication, nodal segments of 5 mm from *in vitro* produced shoots were cultured on Murashige and Skoog (MS), Schenk and Hildebrandt (SH) and Heller (H) media (full strength or diluted to 1/2 or 1/4), with 6-benzylaminopurine (BAP) at concentrations 4.4, 13.3 and 22.2 μM or kinetin (K) at concentrations 4.7, 14.0 and 23.2 μM. The optimum shoot proliferation was on quarter-strength MS medium with 4.4 μM BAP, whereas the maximum number of nodal segments was produced on half-strength MS medium with 4.4 μM BAP. Rooting of shoots was obtained by adding 2.5 - 24.6 μM indole-3-butyric acid (IBA) and broad range of macronutrients; Lloyd and McCown (WPM) and Gresshoff and Doy (GD) media both full strength or diluted to 1/2 were optimum. No rooting was obtained in the presence of α-naphthaleneacetic acid (NAA).

Additional key words: auxins, cytokinins, macronutrients requirement, micropropagation, tissue culture.

Introduction

Myrtus communis L., a circummediterranean bush, is the only European member of family *Myrtaceae*. Use of *M. communis* as a hedge species is widespread in areas with mediterranean climates. Antimicrobial qualities of *M. communis* extracts have

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Abbreviations: BAP - 6-benzylaminopurine; GD - Gresshoff and Doy (1972) medium; H - Heller (1953) medium; IBA - indole-3-butyric acid; K - kinetin; MS - Murashige and Skoog (1962) medium; NAA - α-naphthaleneacetic acid; SH - Schenk and Hildebrandt (1972) medium; WPM - LLoyd and McCown (1981) medium.

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been reported (Salih and Nadir 1984, Garg and Dengre 1988). In order to select desirable properties and for rapid increase of plant populations or germplasm collections, clonal propagation of elite genotypes through *in vitro* culture techniques would be useful (Pierik 1987). In addition, different explant types of *M. communis* are used in our laboratory in order to study recalcitrance during morphogenesis in this woody species. Therefore, we are interested in optimized shoot proliferation protocols that allow us to obtain a great number of aseptic explants, avoiding contamination problems and seasonal variations associated with field material.

Some species of the *Myrtaceae* have been micropropagated (*e.g.* Speer 1993). Micropropagation of adult *M. communis* has been reported by Khosh-Khui *et al.* (1984) using plants from Iran. These authors found high rates of shoot proliferation when 6.7 μM BAP plus 0.5 μM NAA were added to half-strength MS. Recently, Nobre (1994) has reported high rates of shoot multiplication of *M. communis* plants of Portugal from nodal explants placed horizontally on Quoirin and Lepoivre medium with 0.25 μM NAA plus 8.8 μM BAP. Although subspecific variation in *M. communis* has not been described, differences in *in vitro* responses are usual among different genotypes of a plant species. For instance, Korban *et al.* (1992) reported great differences in the effect of growth regulators supply on regenerative responses from different *Malus* × *domestica* genotypes. Coleman and Ernst (1990) found a significant interaction between genotype and cytokinin concentration on *in vitro* shoot regeneration of *Populus deltoides*. Selby and Harvey (1990) reported that various genotypes of *Picea sitchensis* showed different responses to a range of total inorganic nitrogen supply and NO_3^-/NH_4^+ balances.

The aim of this work was to find the mineral and hormonal requirements for rapid and efficient *in vitro* multiplication and rooting of the East Spanish population of *M. communis* used in our laboratory, and to determine whether this population exhibits the same or different responses in comparison to other genotypes previously studied by other authors.

Materials and methods

Shoot establishment: Nodal segments (10 - 20 mm) were collected from adult *M. communis* plants growing in the field. Leaves were clipped off and the explants were surface sterilized with 70 % (v/v) ethanol for 1 min, followed by 6 % $Ca(ClO)_2$ for 20 min, and then rinsed three times with sterile distilled water. The initial explants were placed for 5 - 7 d on a preincubation medium containing only 30 g dm^{-3} saccharose and 0.8 % agar to prevent the oxidation of phenolic compounds released at the beginning of culture. The explants were then transferred to an establishment medium for three subcultures (of 60 d each) until sufficient material was available for study. The establishment medium was MS macronutrients with 5 mg dm^{-3} thiamine, 100 mg dm^{-3} myo-inositol, 3.6 μM BAP, 317.2 μM phloroglucinol, 20 g dm^{-3} saccharose and 0.8 % agar.

Shoot multiplication: Multiplication studies were carried out with nodal segments (5 mm long, each containing a pair of axillary buds) obtained from the stock cultures on establishment medium. Macronutrients of MS, SH, and H, in combination with MS micronutrients were tested (at full strength or reduced to 1/2 or 1/4 strength). Two cytokinins were added to media: BAP (4.4, 13.2 and 22.2 μM) or K (4.7, 14.0 and 23.2 μM). All media were supplemented with 2 mg dm^{-3} glycine, 100 mg dm^{-3} myo-inositol, 10 mg dm^{-3} thiamine, 0.5 mg dm^{-3} nicotinic acid, 0.5 mg dm^{-3} pyridoxine and 30 g dm^{-3} saccharose. Since both Khosh-Khui *et al.* (1984) and Nobre (1994) found that low levels of NAA improved shoot multiplication in this species, an additional experiment was performed in which MS/2 medium with 4.4 μM BAP was supplemented with 0.5 μM NAA.

After 8 weeks of culture, dead explants were discarded. In each surviving explant, the number of shoots of at least 2 mm length and the maximum shoot length were quantified. In order to evaluate the number of explants available to be easily excised and subcultured in routine shoot proliferation protocols, the number of nodal segments of 5 mm that can be obtained by fragmentation of shoots was also measured.

Shoot rooting: For rooting studies, individual shoots (5 - 10 mm long each) were removed from the clusters growing on the best proliferation medium and then transferred to 5 different media: macronutrients of MS, SH, H, GD and WPM in combination with MS micronutrients. Macronutrients were used at full strength or 1/2 strength. Two auxins were added to media: NAA (2.4, 5.0, 14.9 and 24.7 μM) or IBA (2.5, 4.9, 14.7 and 24.6 μM). All media were supplemented with 2 mg dm^{-3} glycine, 100 mg dm^{-3} myo-inositol, 10 mg dm^{-3} thiamine, 0.5 mg dm^{-3} nicotinic acid, 0.5 mg dm^{-3} pyridoxine and 30 g dm^{-3} saccharose. During 4 weeks, percentage of rooted shoots was measured every 5 d.

Culture conditions: Culture media were adjusted to pH 5.7 - 5.8 before adding 0.8 % agar (*Probus*), and they were sterilized by autoclaving at 121 °C for 20 min. The explants were incubated in 55 × 75 mm glass flasks, 5 explants per flask, each containing 30 cm^3 of medium. The flasks were capped with aluminium foil. Each treatment was applied to 25 explants.

All cultures were maintained under a 14 h photoperiod with irradiance of 60 μmol m^{-2} s^{-1}, at day/night temperature 24/18 ± 1 °C.

Results

Shoot establishment: The initial explants established in culture from field plants exuded phenolic compounds to the medium. When these explants were placed on a standard medium with mineral salts, the oxidation of phenolic exudates was evidenced by a great browning of the medium, that caused inhibition of both explant survival and growth (data not shown). However, a pre-incubation period of 5 - 7 d of field explants on preincubation medium prevented the browning of the medium.

When the explants were transferred from preincubation medium to establishment medium, all survived and the buds sprouted within 10 - 15 d.

Shoot proliferation: Death of some explants was observed during the first weeks of culture. In particular, explant survival was low on media H/2 and H/4.

Kinetin induced very low shoot proliferation rates, that were not significantly higher than controls. On the other hand, addition of BAP resulted in a significant effect on number of shoots per explant (Fig. 1). The maximum mean yield of shoots per explant (25.5 ± 4.7) was found on MS/4 medium with 4.4 μM BAP, followed by MS/2 medium with 22.2 μM BAP (19.5 ± 2.5) or 4.4 μM BAP (18.6 ± 2.5).

Macronutrient composition was a significant source of variation on shoot multiplication (*F* value 23.46; $P < 0.0001$). MS was better than SH, and SH was better than H (Fig. 1).

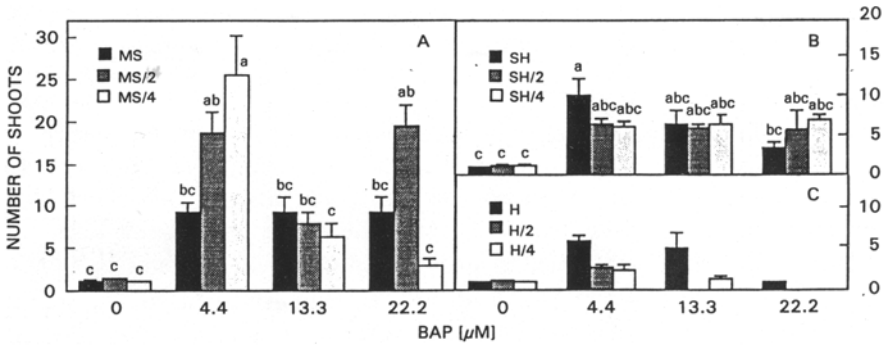

Fig. 1. Mean (± S.E.) number of shoots of at least 2 mm long obtained per explant after 8 weeks of culture on shoot proliferation media containing MS (*A*), SH (*B*) and H (*C*) macronutrients and 0, 4.4, 13.3 and 22.2 μM BAP. In each diagram, means followed by the same letter are not significantly different at the 0.05 level (Tukey test).

When MS macronutrients were used (Fig. 1A), a strong interaction was detected between the two sources of variation "Salt strength" and "BAP concentration" (*F* value of interaction 6.01; $P < 0.0001$). In both MS and MS/2 media no significant difference was found between number of shoots in the presence of BAP; whereas in MS/4 medium 4.4 μM BAP induced significant higher response than 13.3 or 22.2 μM BAP. Therefore, the best BAP supply was dependent on MS salt strength and *vice versa* (Fig. 1A).

When SH macronutrients were used (Fig. 1B), no significant effect of salt strength on number of shoots was obtained, and the influence of BAP concentration was minimum. Only SH medium with 4.4 μM BAP was significantly better than SH with 22.2 μM BAP or media without cytokinins.

On H medium shoot proliferation was slow (Fig. 1C) and explants became yellowish and necrotic. In addition, no explant survived on three of these media (H/2

with 13.3 or 22.2 μM BAP, H/4 with 22.2 μM). So, effects of salt strength and cytokinin concentration in these media were not analysed statistically.

During the shoot proliferation stage, shoot elongation was poor in all cultures (MS, SH, H and their 1/2 and 1/4 dilutions). The mean maximum shoot length never reached 15 mm, and it was not significantly altered by the addition of cytokinins (data not shown).

Table 1. Number (mean ± S.E.) of nodal segments of 5 mm obtained per explant after 8 weeks of culture on different shoot proliferation media.

Culture medium	BAP [μM]				K [μM]		
	control	4.4	13.3	22.2	4.7	14.0	23.2
MS	0.7 ± 0.2	2.8 ± 0.4	2.5 ± 0.5	1.6 ± 0.4	1.4 ± 0.3	2.6 ± 0.4	1.5 ± 0.6
MS/2	1.5 ± 0.2	6.7 ± 0.9**	1.7 ± 0.5	1.3 ± 0.6	1.0 ± 0.3	0.6 ± 0.4	1.5 ± 0.5
MS/4	1.4 ± 0.3	2.4 ± 0.5	0.9 ± 0.3	0.6 ± 0.4	0.4 ± 0.2	0.7 ± 0.3	1.1 ± 0.4
SH	1.3 ± 0.3	2.5 ± 0.7	2.7 ± 0.8	1.6 ± 0.3	1.8 ± 0.3	1.9 ± 0.3	1.2 ± 0.3
SH/2	1.7 ± 0.4	2.9 ± 0.4	2.5 ± 0.4	2.1 ± 0.6	1.4 ± 0.2	1.9 ± 0.3	2.1 ± 0.2
SH/4	1.3 ± 0.4	3.3 ± 0.3	3.2 ± 0.7	2.4 ± 0.3	1.9 ± 0.3	2.2 ± 0.2	1.9 ± 0.2
H	1.4 ± 0.3	1.1 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	1.7 ± 0.3	2.8 ± 0.4	1.7 ± 0.3
H/2	0.6 ± 0.3	0.2 ± 0.2	n.s.	n.s.	2.0 ± 0.3	2.7 ± 0.3	2.1 ± 0.3
H/4	0.6 ± 0.3	0.3 ± 0.2	0.0 ± 0.0	n.s.	1.3 ± 0.4	1.2 ± 0.3	0.6 ± 0.3

** - value significantly different at the 0.05 level (Tukey test). n.s. - explants did not survive

The number of nodal segments of 5 mm available to be excised and subcultured for multiplication was highest on MS/2 medium with 4.4 μM BAP (Table 1), because it induced both high multiplication and acceptable elongation responses. This medium was selected to produce shoots for rooting studies. When the optimum medium for shoot multiplication reported by Khosh-Khui *et al.* (1984) was tested

Table 2. Percentage of rooted shoots after 4 weeks of culture on rooting media: effect of mineral salts and IBA concentration. Means followed by the same letter are not significantly different at the 0.05 level (Tukey test with arcsin transformation of percentages). Nontransformed data are presented.

Culture medium	IBA [μM]				
	0	2.5	4.9	14.7	24.6
MS	12 d	40 bcd	4 d	16 d	28 cd
MS/2	32 bcd	32 bcd	12 d	44 bcd	36 bcd
SH	8 d	4 d	12 d	36 bcd	48 bcd
SH/2	36 bcd	4 d	12 d	36 bcd	48 bcd
H	20 d	40 bcd	44 bcd	76 abc	4 d
H/2	8 d	20 d	44 bcd	92 a	16 d
GD	28 cd	84 ab	88 a	56 abcd	28 cd
GD/2	52 abcd	56 abcd	80 ab	76 abc	64 abc
WPM	28 cd	84 ab	68 abc	64 abc	56 abcd
WPM/2	8 d	72 abc	88 a	88 a	96 a

(MS/2 with 0.5 μM NAA plus 6.7 μM BAP), it induced significant lower values of both number of shoots (6.4 ± 1.4) and segments (2.6 ± 0.7) as compared with our optimum medium.

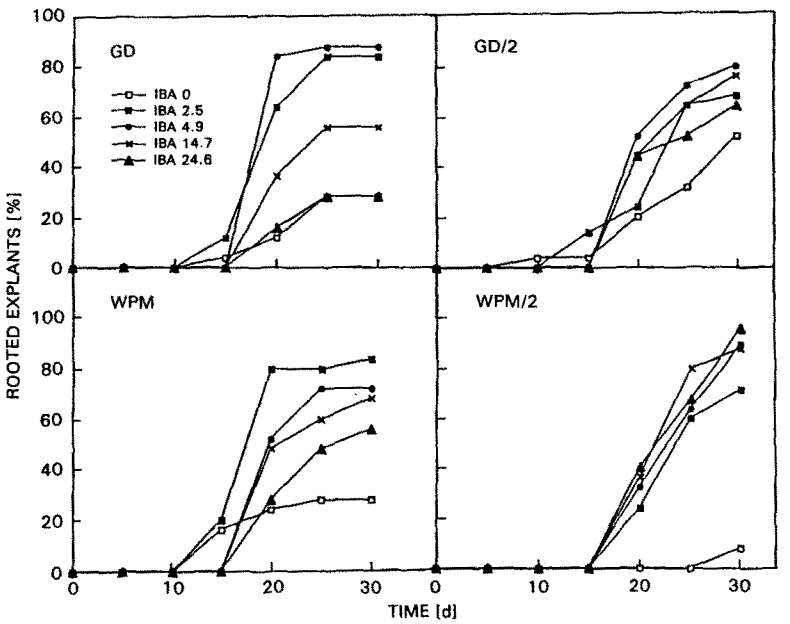

Fig. 2. Progression of the percentage of rooted shoots during the first 4 weeks of culture on the best rooting media (WPM, WPM/2, GD, GD/2) in the presence of 0, 2.5, 4.9, 14.7 and 24.6 μM IBA.

Shoot rooting: Explant survival was 100 % in all treatments and no callus was formed at the base of explants. When NAA was added root formation did not ocur, but IBA induced rooting in all media (Table 2). Low rooting percentages after 4 weeks of culture were found on the majority of media without growth regulators. High rooting percentages (Table 2) were found on media with GD or WPM (full or half strength) macronutrients. Lowest rooting responses appeared on MS and SH (full or half strength) media. No root initiation ocurred during the first two weeks of culture, with most rooting ocurring during the third week (Fig. 2). The pattern of rooting progression was similar for all media.

Discussion

In this study, *M. communis* shoot proliferation has been achieved with 4.4 - 22.2 μM BAP in a broad range of basal media. All results are not in agreement with previous reports: *1*) Khosh-Khui *et al.* (1984) found that full-strength MS salts supressed explant growth and the tips of the leaves were burned, whereas in our study all explants on full strength MS were green and healthy; *2*) both Khosh-Khui *et al.*

(1984) and Nobre (1994) reported that addition of NAA (0.5 μM for plants of Iran and 0.25 μM for plants of Portugal) improved shoot multiplication, whereas in our study 0.5 μM NAA significantly decreased both number of at least 2 mm shoots and number of 5 mm segments.

Lack of repeatability in *in vitro* propagation responses may result from genetic heterogeneity (Juncker and Favre 1989). Khosh-Khui *et al.* (1984) seemed to test shoot proliferation media using tips excised from vegetative shoots growing in the field whereas in the present study and in the report of Nobre (1994) the explants were obtained from a stock collection growing *in vitro*.

On media containing H macronutrients (full, 1/2 or 1/4 strength) very low explant survival and proliferation responses were found, and the explants that survived seemed yellowish and necrotic. In this medium total nitrogen supply is very low and ammonium ions are absent. The supply of nitrogen as NH_4^+ may play an important role on shoot proliferation in these cultures. In addition, NH_4^+/NO_3^- ratio is higher in MS (20.6/39.4 mM) than SH (2.6/24.7 mM) medium, and we have found better shoot proliferation on MS (full strength or diluted) media. Presence of both ammonium and nitrate ions as nitrogen supply, and appropriate balances of them, have been reported to be neccesary in order to improve shoot proliferation and morphogenetic responses in a wide range of species (Samartin 1989, Kaul and Hoffman 1993, Misra and Chaturvedy 1992). In this paper, however, the possibility that other aspects of ionic composition of the basal medium may influence shoot proliferation cannot be excluded (as the presence of relatively high levels of both Na^+ and Cl^- in H medium, or Ca^{2+} in MS medium).

Among all media tested, shoot elongation was found to be low during proliferation stage. Poor elongation have been described in another species of *Myrtaceae*, *Syzygium aromaticum*. In this species, Mathew and Hariharan (1990) found that shoots proliferating from axillary buds failed to elongate on MS/2 medium with 2.2 - 13.3 μM BAP, either in the presence or in the absence of NAA.

In the rooting stage, results are in strong disagreement with previous reports. Both Khosh-Khui *et al.* (1984) and Nobre (1994) obtained high percentages of rooting with NAA, whereas in our case NAA never induced root formation. In addition, Khosh-Khui *et al.* (1984) found 2.2 μM BAP to be necessary for explant survival during the rooting stage, whereas Nobre (1994) has described succesful rooting in the absence of cytokinin. Our data confirm the result reported by Nobre. Therefore, requirements of growth regulators for *in vitro* rooting may be strongly genotype dependent in this species.

For rooting, Khosh-Khui *et al.* (1984) tested only MS and MS/2 media, and Nobre (1994) used Quoirin and Lepoivre medium. In our study we have tested MS, SH, H, GD and WPM media (full strength or diluted), and GD or WPM were the best. WPM medium has been reported to be superior to MS (full strength or diluted) for rooting of woody species (Orlikowska 1992).

Although the time between subcultures (8 weeks) during the multiplication stage is longer than time required for other woody species, the optimum protocol described in this paper is suitable for a large scale *in vitro* propagation of the East Spanish population of *Myrtus communis* studied in our laboratory.

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