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Micropropagation of *Rosmarinus officinalis* L.*

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Abstract. Single-node stem segments of *Rosmarinus officinalis* L. var. *genuina* forma *erectus* proved better explants than shoot tips (ca. 2 cm long) for establishment of field-grown plants in aseptic cultures. Benzylaminopurine was far more effective than kinetin for shoot induction in shoot tips excised from aseptically-grown plants. Maximum numbers of shoot buds (ca. 14) were formed per explant at 0.2 mgl^{-1} benzylaminopurine in 30 days. After further growth of isolated shoots and treatment with 0.25 mgl^{-1} indolepropionic acid for 7 days, 80% shoots produced roots. In vitro raised plantlets were successfully grown in soil to plants. About 5,000 plants could be produced from a single nodal segment in 1 year.

Introduction

Rosmarinus officinalis (rosemary) described as a 'miracle herb' [11] is the source of rosemary oil of commerce. The oil is used widely in high quality perfumes, cosmetics and in certain medicinal preparations [2, 11, 12]. There are several varieties/clones of rosemary which vary in their essential oil content [8]. A method for rapid multiplication of elite clones by tissue culture would be useful. The tissue culture of some members of Labiatae, viz., *Mentha* spp. [10, 16], *Pogostemon cablin* [9, 15], *Thymus vulgaris* [7] and *Ocimum sanctum* [1] has been reported but there appears to be no tissue culture studies in rosemary. We report here the in vitro multiplication and clonal propagation of rosemary.

Materials and methods

Elite plants of *Rosmarinus officinalis* L. var. *genuina* forma *erectus* grown in Solan (Simla) comprised the experimental plant material. Shoot tips as well as single-node stem segments ca. 2 cm in length (Figure 1a) were excised from field-grown plants. Their basal cut ends were sealed with molten paraffin and the explants were treated with a liquid detergent Teepol (BDH)

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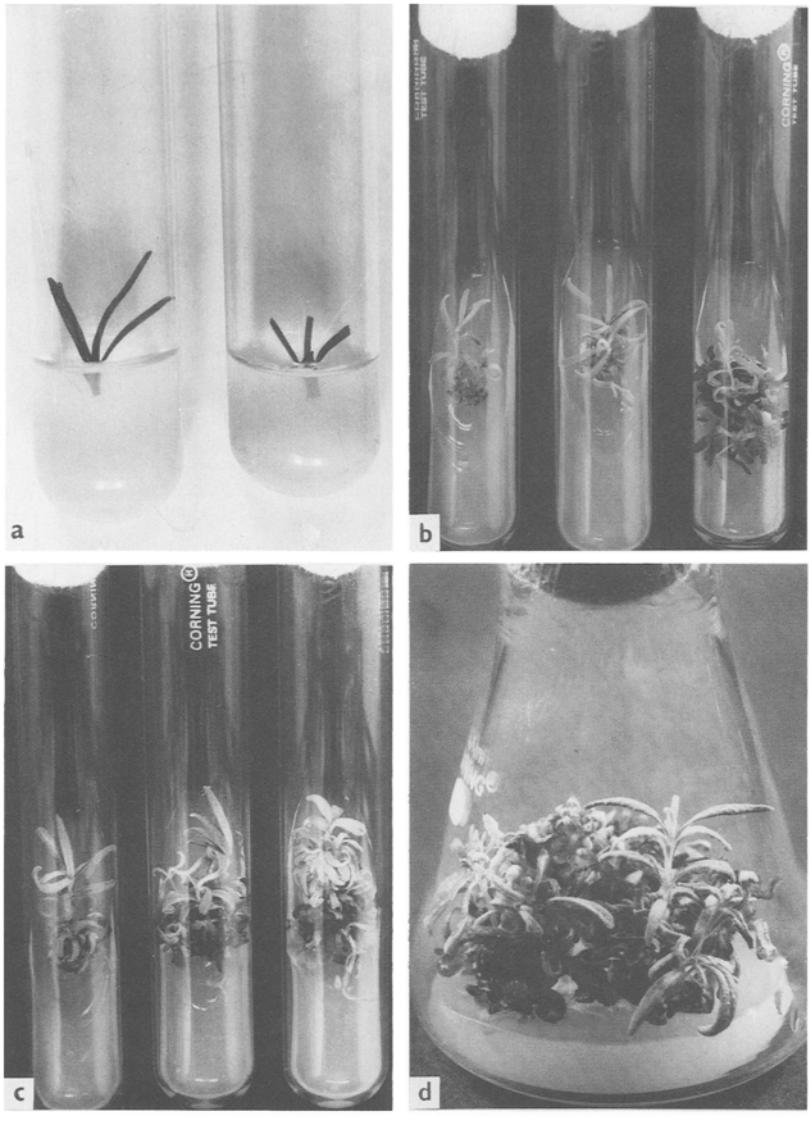

Figure 1. Cultures of *Rosmarinus officinalis* L. (a) Explant of a shoot tip (left) and a single-node stem segment (right) taken from field-grown plant, respectively. (× 1.1) (b, c) Formation of shoot-buds in excised shoot tips and their development into shoots in treatments containing 0.05, 0.1 and 0.2 mg l^{-1} BAP (from left to right) with MS1 (b) and MS2 (c), respectively. (× 0.8 each) (d) Proliferating and developing shoots after one subculture of shoot tips inoculated along with differentiated shoot buds. (× 1.28)

solution (5%) for 5 min followed by a 2 sec dip in 95% ethanol. Surface sterilization was in 0.1% aqueous solution of $HgCl_2$ for 3–5 min (shoot tips) or 5–10 min (nodal segments). After surface sterilization, approximately 2 mm was removed from the cut ends of the explants and they were thrice washed with sterile distilled water with 5 min duration each. Explants were then kept for ca. 30 min in either sterile distilled water or in polyvinylpyrrolidone (PVP, soluble, M.W. 44,000) solution (100 mg l^{-1}).

The pH of the media used was adjusted to 5.8. Media were sterilized by autoclaving at 1.08 kg/cm^2 for 15 min. The cultures were grown under 3 klux light from Phillips day light fluorescent tubes for 14 h daily at 27 ± 1 °C.

White's [17] medium supplemented with 0.1 mg l^{-1} indoleacetic acid (IAA), 5.0 mg l^{-1} gibberellic acid (GA), 5.0 mg l^{-1} ascorbic acid and 20 mg l^{-1} glutathione, named as WM, was used for establishment of rosemary plants in aseptic condition. Two modifications of Murashige and Skoog's [14] medium (MS1 and MS2) supplemented with 10 mg l^{-1} adenine sulphate (AdS), 0.1 mg l^{-1} IAA, 50 mg l^{-1} malt extract (ME) and 20,000 sucrose were used as the basal media for proliferation of shoot tips. The media MS1 and MS2 differed from the Murashige and Skoog's [14] medium in having (mg l^{-1}): 1000 and 1500 NH_4NO_3, 200 and 1500 KNO_3, 300 and 400 $CaCl_2 \cdot 2H_2O$, 200 and 360 $MgSO_4 \cdot 7H_2O$, 80 and 150 KH_2PO_4, 22.4 and no change Na_2-EDTA, 16.7 and no change $FeSO_4 \cdot 7H_2O$, 0 and no change m-inositol and 0.1 and 0.1 pyridoxine-HCl, respectively. Different concentrations of benzylaminopurine (BAP) or kinetin (Kn) were tested for induction of shoot buds. Before rooting, isolated shoots were grown in MS2 supplemented with 10–50 mg l^{-1} AdS, 2.5–10 mg l^{-1} GA and 2–4% sugar along with 0.05 mg l^{-1} IAA in the presence or absence of m-inositol (100 mg l^{-1}).

IAA, indolepropionic acid (IPA), β-indolebutyric acid (IBA) and α-naphthaleneacetic acid (NAA) at 0.25 and 0.5 mg l^{-1} concentrations in the medium were tested for root induction in isolated shoots. Three methods for the transfer of rooted shoots with no callus and those with callus to soil were used: 1) transfer to manured soil (garden soil : leaf mould 3:1), 2) transfer to Vermiculite with added nutrient solution, and 3) culture in liquid inorganic salt solution for 15 days using the procedure reported earlier [3] before transplantation to soil. Plantlets were covered by transparent polyethylene chambers for 10 days after transfer to soil in order to prevent desiccation and were kept under culture room conditions.

Results and discussion

In medium WM all the shoot tips, whether kept in distilled water or PVP solution before inoculation, turned brown within 7 days of incubation. Similarly, single-node stem segments stored in distilled water prior to inoculation gradually turned brown in the medium within 10 days. Ca. 10% of

nodal segments, soaked in PVP solution, remained green in culture and produced axillary shoots after 25–30 days. When the surviving stem segments were subcultured in the same medium, little growth of developing axillary shoots occurred. On the other hand, the axillary shoots grew well in MS1 supplemented with 0.1 $mg\,l^{-1}$ IAA and 5 $mg\,l^{-1}$ GA. It is a common experience that White's [17] medium because of its low salt concentration is suitable for seed germination, excised root growth and initial culture of shoot explants where callusing is a disadvantage and Murashige and Skoog's [14] medium with its high salt concentration and because of the presence of NH_4NO_3 is generally more suitable for shoot proliferation [6].

One cm long shoot tips excised from aseptically regenerated shoots proliferated to different extents in the two basal media MS1 and MS2 in response to Kn or BAP. The number of shoots obtained in the presence of BAP in MS1 and MS2 are presented in Table 1. Compared to BAP, there was very little proliferation of shoots in 0.05, 0.1 and 0.2 $mg\,l^{-1}$ concentrations of

Table 1. Differentiation of shoot buds on shoot tip explants of *Rosmarinus officinalis**. No. of shoot buds per explant (mean and standard variation of 5 replicates)

Treatment ($mg\,l^{-1}$)	Incubation (days)		
	10	20	30
Control – MS1 (basal medium)	0	0	0
BAP 0.05	2.2 ± 0.49	4.4 ± 0.40	5.8 ± 0.37
BAP 0.1	2.6 ± 0.50	4.8 ± 0.37	6.4 ± 0.50
BAP 0.2	1.8 ± 0.49	5.6 ± 0.74	13.6 ± 0.92
Control – MS2 (basal medium)	0	0	0
BAP 0.05	2.2 ± 0.38	3.0 ± 0.32	3.6 ± 0.51
BAP 0.1	2.6 ± 0.51	4.8 ± 0.38	6.0 ± 0.55
BAP 0.2	1.6 ± 0.40	5.6 ± 0.81	6.8 ± 0.73

*Similar results were obtained on repetition of the experiment.

Kn, i.e., a maximum of 1, 3 and 3 shoots, respectively, with MS1 and 1, 1 and 2 shoots, respectively, with MS2 were produced in 30 days. With a number of other plant species Kn is less effective than BAP [13]. With both the basal media, 0.1 $mg\,l^{-1}$ BAP was found most suitable for shoot formation. With 0.2 $mg\,l^{-1}$ BAP an increased number of shoots formed but was accompanied by callus formation. For clonal propagation, shoot formation should be free from callusing because the callus may be genetically unstable [5]. In the same concentrations of BAP, the number of shoots formed was higher with MS1 than with MS2, whereas shoot growth was better with MS2 (Fig. 1b and c). Shoots appeared at the same time in BAP-treatments irrespective of the basal medium used, i.e., after about 10 days of incubation. Inorganic salts in the medium have been reported to greatly

modify the effect of growth substances [4]. Formation of shoots was continuous and nonsynchronous. While, earlier formed shoot buds developed into shoots, new buds continued to appear on the explant. The highest number of shoot buds was obtained after 30 days of incubation. An average of 14 shoot buds formed on MS1 containing 0.2 $mg\,l^{-1}$ BAP. All the shoot buds and shoots from an explant were green and healthy in appearance up to about 30 days of incubation, beyond which browning of leaves started. Before browning occurred, the original shoot explant along with all the shoots and buds was subcultured on to MS1 medium containing 1500 $mg\,l^{-1}$ NH_4NO_3 and 5 $mg\,l^{-1}$ each of ascorbic acid and cysteine. The cultures remained green for up to 45 days in this medium (Fig. 1d).

The shoots isolated from cultures developed well on MS2 containing 25 $mg\,l^{-1}$ AdS, 5.0 $mg\,l^{-1}$ GA, 0.05 $mg\,l^{-1}$ IAA and 3% sucrose but devoid of m-inositol.

In 0.25 $mg\,l^{-1}$ IPA, about 80% shoots, measuring approx. 3.0 cm in length, rooted within 7 days. Roots were thin and no callus was formed. However, if the shoots were allowed to remain in the same IPA-treatment beyond 15 days, the roots became thicker and the base of the rooted shoots callused. When shoots with just visible roots were transferred to an auxin-free medium, the roots grew normally. IAA was less suitable than IPA for rooting. Though the percentage of rooting and the time taken in root induction was the same with both the auxins, roots produced in IAA were brownish from the beginning and restricted in growth. NAA and IBA, proved to be ineffective for rooting at 0.25 and 0.5 $mg\,l^{-1}$.

When transplanted to sterile soil about 60% of rooted shoots survived. Culture of rooted shoots in liquid medium before transfer to soil or transplantation into Vermiculite gave only 20% survival. Shoots with roots accompanied or unaccompanied with callusing gave similar transplantation success. Plants in sterilized potted soil grew normally under glass house conditions and looked alike. It took about 1 month to get a 3 to 4 cm long plantlet in soil from the time of rooting of shoots in vitro.

About 5,000 plants could be produced from a survived nodal explant within 1 year from the time of its collection from a field-grown elite plant. The initial culture of nodal explants required 3 months to produce a culture of proliferating and developing shoots. From this approximately 10 shoots were obtained for rooting and the remaining shoots gave two new cultures. Each in turn provided 10 shoots after a month and so on.

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