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Research note

Micropropagation of *Thymus piperella*

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Abstract

Explants from aseptically germinated seeds of *Thymus piperella* L. were induced to form shoots on modified Murashige and Skoog medium, the best yield being 5.1 shoots per explant when the medium contained 6.6 μM BA plus 2.8 μM IAA. Shoots could be rooted on the same basal medium supplemented with 2.8 μM IAA, and 71% of the plantlets were successfully acclimatized.

Abbreviations: BA – benzyladenine, CMS – modified MS culture medium, IAA – indoleacetic acid, MS – Murashige & Skoog (1962) culture medium, NAA – α-naphthaleneacetic acid

Thymus piperella L. (Labiatae), a species endemic to eastern Spain, is valued for its essential oil (Adzet & Passet 1976) and use as a culinary herb (Alcaraz et al. 1989). Because demand from the flavour industry is increasing, the development of an in vitro system for the propagation of elite clones is desirable. Since previous research on *Thymus* has been restricted to *Thymus vulgaris* (Furmanowa & Olszowska 1980, 1992; Olszowska 1982; Olszowka & Furmanowa 1987; Lê 1989), we initiated this study to develop a micropropagation technique for the distinctive *Thymus piperella*, a broad-leaved, glabrous and more herbaceous species, placed by taxonomists in a different section of the genus (Morales 1986).

Thymus piperella seeds were collected and kept at 8°C until surface disinfestation. This consisted of soaking the seeds in distilled water with a few drops of Tween 20 (5 min), then in a 7% (w/v) solution of calcium hypochlorite (20 min), rinsing with sterile water, soaking in 70% (v/v) ethanol (5 min) followed by three rinses of 5, 10 and 15 min in sterile water.

The seeds were germinated on CMS medium (Collet 1985) with full-strength inorganic salts plus 6.0 $g\,l^{-1}$ Difco Bacto-agar and without sucrose. CMS salts differ from MS salts (Murashige & Skoog 1962) by

- the presence of $NH_4H_2PO_4$ and $Ca(NO_3)_2$ instead of NH_4NO_3, KH_2PO_4 and $CaCl_2$, which means a substantial reduction in nitrogen, mainly NH_4^+,
- an almost 50% reduction in KNO_3 and $MgSO_4$, and
- a 60% reduction in FeNaEDTA.

Seedlings were kept on 5 ml of medium in 2 × 15 cm glass tubes closed with translucent plastic caps for 2 months with a 16-h photoperiod (40 μmol m^{-2} s^{-1}, Sylvania Gro-lux fluorescent tubes) at 25 ± 2°C.

The most vigorous plantlets in the tubes were transferred to larger glass vessels (300 ml volume, closed with translucent plastic screw caps) containing 50 ml of CMS basal medium (7.5 $g\,l^{-1}$ agar, 20 $g\,l^{-1}$ sucrose). As reported by Lê (1989) for *Thymus vulgaris*, we found a stressed morphology in the explants grown on basal medium (smaller leaves and shorter internodes than in vivo), which increased with increasing concentrations of BA (6.6–8.9 μM). To produce a shoot population for further experimentation without this stress morphology, shoot explants were subcultured monthly for 7–8 months on CMS basal medium supplemented with 2.2 μM BA, the roots being excised each time.

Shoot explants 2.5–3.5 cm long, containing an apical bud and 3 nodes, were excised and cultured on basal

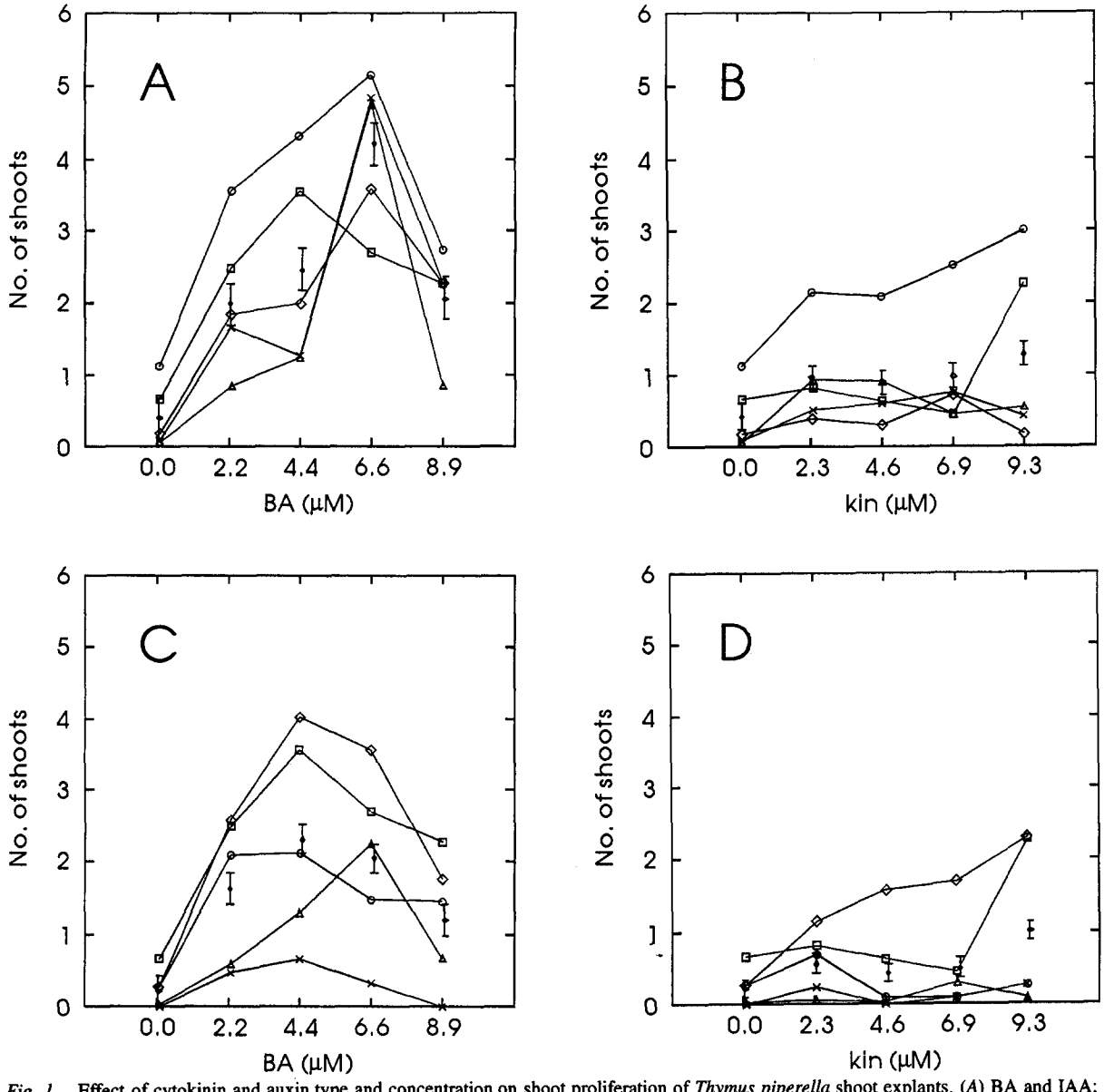

Fig. 1. Effect of cytokinin and auxin type and concentration on shoot proliferation of *Thymus piperella* shoot explants. (*A*) BA and IAA; (*B*) kinetin and IAA; (*C*) BA and NAA; (*D*) kinetin and NAA. Concentrations of IAA were 0.0 (□), 1.1 (◇), 2.8 (○), 8.5 (△) and 17.1 (×) μM, and of NAA were 0.0 (□), 1.0 (◇), 2.7 (○), 8.0 (△) and 16.1 (×) μM. Each data point is the mean of 28 explants (four replications, each based on seven explants). Data analyzed with a 'Friedman two-way nonparametric' analysis of variance. Significance levels for each cytokinin concentration stated with Tukey's LSD test (rejection level = 0.05). LSD values for comparison (shown as ± LSD for mean of auxin concentrations at each cytokinin concentration) are: A: 0.58; B: 0.33; C: 0.42; D: 0.26.

CMS medium containing growth regulators in various combinations (IAA × BA, IAA × kinetin, NAA × BA and NAA × kinetin) and concentrations (0, 2.2, 4.4, 6.6 and 8.9 μM BA; 0, 2.3, 4.6, 6.9 and 9.3 μM kinetin; 0, 1.1, 2.8, 8.5, and 17.1 μM IAA; 0, 1.0, 2.7, 8.0 and 16.1 μM NAA). In this way, 100 treatments, 25 for each 'auxin × cytokinin' combination, were tested.

Seven explants per vessel (four vessels per treatment) were cultured for 30 days. The number of shoots produced by each explant was then recorded, and the root development per explant was classified into four

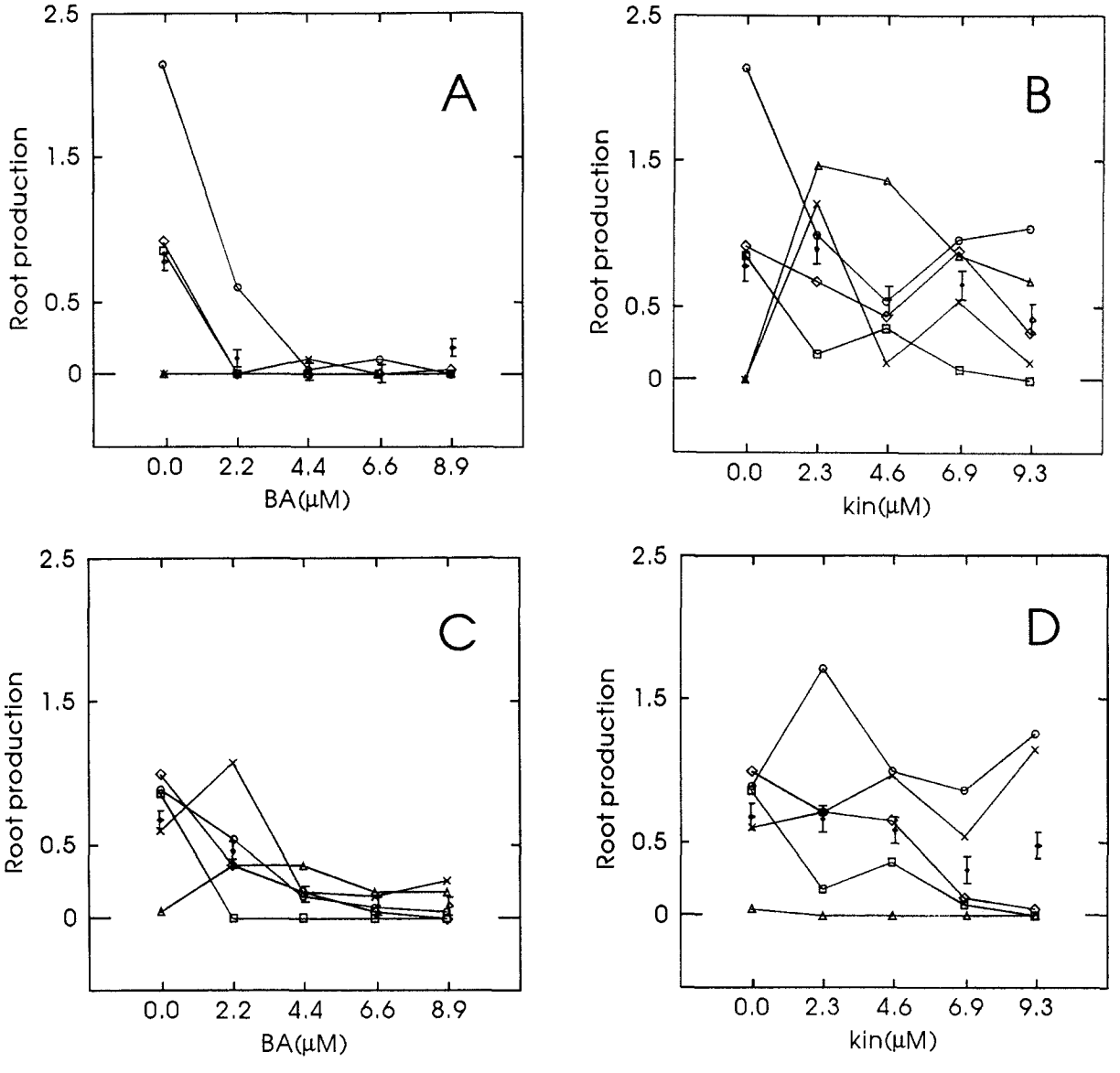

Fig. 2. Effect of cytokinin and auxin type and concentration on root production of *Thymus piperella* shoot explants. (*A*) BA and IAA; (*B*) kinetin and IAA; (*C*) BA and NAA; (*D*) kinetin and NAA. Concentrations of IAA were 0.0 (□), 1.1 (◇), 2.8 (○), 8.5 (△) and 17.1 (×) μM, and of NAA were 0.0 (□), 1.0 (◇), 2.7 (○), 8.0 (△) and 16.1 (×) μM. Each data point is the mean of 28 explants (four replications, each based on seven explants). Data analyzed with a 'Friedman two-way nonparametric' analysis of variance. Significance levels for each cytokinin concentration stated with Tukey's LSD test (rejection level = 0.05). LSD values for comparison (shown as ± LSD for mean of auxin concentrations at each cytokinin concentration) are: A: 0.12; B: 0.20; C: 0.11; D: 0.17.

groups according to the quantity and length of the roots (0 = no root production; 1 = roots < 0.5 cm long; 2 = 1–2 roots >= 1 cm long or any number of roots 0.5–1 cm long; 3 = 3 or more roots >= 1 cm long). This resulted in a number from 0 to 3 for each explant. Since the results did not show a normal distribution pattern and could not therefore be adequately transformed, statistical analysis of these four randomized blocks, with four replicates (seven subplots each) for each of the 25 treatments per block, was carried out with a 'Friedman two-way nonparametric' analysis of variance.

For in vivo acclimatization, 100 plantlets were cultured for 20 days in CMS medium with half-strength macronutrients using the growth regulator combina-

tion that was found previously to have the best effect on root development. After that, they were transferred to sterilized compost (peat:vermiculite, volume 1:1). Plantlets were sprayed with a 50% glycerol/diethyl ether mixture to reduce transpiration, and kept in pots inside plastic bags for 15 days in a greenhouse. The bags were gradually opened and the plants were finally transferred to soil.

The shoot promoting ability of BA (maximum at 4.4–6.6 μM) was greater than that of kinetin (maximum at 9.3 μM). Similarly, IAA (maximum at 2.8 μM) showed a greater shoot-promoting ability than NAA (maximum at 1.0 μM) (Fig. 1).

Root development was inhibited more by BA than by kinetin, in the presence of either IAA or NAA (Fig. 2). Best root development was found with 2.8 μM IAA and no cytokinin; this treatment was used for the acclimatization process, which was successfully achieved by 71% of the plantlets.

The results concerning the effects of cytokinins on shoot promotion and development do not agree with those of Furmanowa & Olszowska (1992), who found that the number of nodes per shoot decreased as the concentrations of BA and kinetin rose. We, on the other hand, found a peak with BA, while the highest yield occurred at the highest concentration of kinetin. Since their assessment of multiplication was based on nodal sections whereas ours was based on shoot proliferation, our conclusions as to the optimal medium for *Thymus piperella* differ from theirs for *Thymus vulgaris*. However, like Furmanowa & Olszowska (1992), we found that root production was progressively inhibited by increasing concentrations of cytokinin.

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