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Micropropagation of *Cupressus sempervirens* L. and *Chamaecyparis lawsoniana* (A. MURR.) PAR.

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Abstract

Shoots from 18-month-old seedlings of *Cupressus sempervirens* and *Chamaecyparis lawsoniana* were established in

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vitro on modified MURASHIGE and SKOOG medium. Proliferation of axillary shoots occurred without addition of benzyladenine, although a significant increase in numbers of shoots resulted on addition of 0.001 mg.l⁻¹ to 1.0 mg.l⁻¹ benzyladenine. Following conditioning on a growth regulator-free medium for 28 days, 95% of *C. sempervirens* shoots rooted on ½ strength medium containing 1% sucrose and 0.5 mg.l⁻¹ indole butyric acid. Similar levels of rooting were recorded with *Chamaecy-*

paris lawsoniana on the same medium containing 1 mg.l⁻¹ indole butyric acid. *C. sempervirens* shoots were also rooted in non-sterile conditions. Over 80% of rooted shoots of *C. sempervirens* were weaned into glasshouse conditions. The significance of these results in relation to propagation of cypress clones resistant to *Seiridium* canker is discussed.

Key words: *Cupressus sempervirens*, *Chamaecyparis lawsoniana*, micro-propagation, *Seiridium cardinale*.

FDC: 165.44; 174.7 *Cupressus sempervirens*; 174.7 *Chamaecyparis lawsoniana*.

Abbreviations: BA: benzyladenine; H₂O₂: hydrogen peroxide; IBA: indole butyric acid; MSM: Modified MURASHIGE and SKOOG medium; NAA: naphthalene acetic acid; WA: water agar.

Introduction

A serious limitation to the use of *Cupressus* species for timber production and in urban forestry is the incidence of canker caused by *Seiridium cardinale* which, since first being reported from California earlier this century (WAGENER, 1928), has spread to all the areas where *Cupressus* spp. are grown for timber production (PANCONESI and RADDI, 1991). The common European cypress, *C. sempervirens*, a species commonly grown for timber production and amenity purposes in areas with a Mediterranean-type climate (PANCONESI and RADDI, 1991), is moderately susceptible to canker (WAGENER, 1948). Other members of the Cupressaceae are also susceptible to canker, although *Chamaecyparis lawsoniana* (Port Orford Cedar; LAWSON's cypress) appears to be immune (STROUTS, 1973).

Several research workers have demonstrated variation in susceptibility to *Seiridium* canker within provenances and families of *C. sempervirens* (PONCHET and ANDREOLI, 1979; XENOPOULOS, 1990; CROS *et al.*, 1991). Successful exploitation of this resistance could enable the re-introduction of *C. sempervirens* into areas where *Seiridium* canker prevents effective growth.

With the development of suitable techniques, micropropagation could provide a more rapid and efficient method for mass multiplication of resistant genotypes of *Cupressus* spp. than the grafting and softwood cuttings methods currently in use (CALVANESE *et al.*, 1991). Micropropagation has been applied to many coniferous forest tree species, including *Picea abies* (KUNZE *et al.*, 1993), *Pinus taeda* (MOTT and AMERSON, 1982), *P. caribaea* (BAXTER *et al.*, 1989) and *Pseudotsuga menziesii* (ZOGLAUER *et al.*, 1992), and the economics of production

indicate that such a propagation system is feasible for certain species, provided that quite modest genetic gains are achieved (HASNAIN and CHELIAK, 1986). A number of reports have indicated that micropropagation can be successfully applied to *Cupressus* species (FOSSI *et al.*, 1981; HRIB and DOBRY, 1984; FRANCO and SCHWARZ, 1985), although the significance of resistance against *Seiridium* canker has not been considered by these authors.

This paper reports the development of effective micropropagation methods for *Cupressus sempervirens*, and the related species *Chamaecyparis lawsoniana* using juvenile plant material, aimed at the mass-propagation of selected genotypes (e.g. produced by intraspecific or interspecific crosses) with proved resistance (e.g. after inoculation tests) to the fungus.

Materials and Methods

Plant material

Eighteen month-old seedlings of *Cupressus sempervirens* and *Chamaecyparis lawsoniana* were maintained under glasshouse conditions, with no overhead watering, for at least 14 days prior to removal of material for culture. Shoot tips, approx. 50 mm in length, were removed from the top of the crown using a sharp knife, placed inside plastic bags and transferred immediately to the laboratory for surface sterilisation.

Explants were washed for 10 min in running tap water, rinsed in distilled water for 15 min and sterilised by immersion for 10 min in H₂O₂ (30% v/v) containing 0.025% Tween 20 as a wetting agent, followed by 20 min in 20% (v/v) commercial bleach (Domestos) in tap water. Explants were then rinsed in five changes of sterile distilled water, aseptically trimmed to 20 mm in length and inserted into 10 ml culture medium in 30 ml glass jars.

Culture conditions

MSM medium (Sigma), 2.2 g.l⁻¹ in distilled water, supplemented with (per litre) 2 mg glycine, 100 mg inositol, 0.5 mg nicotinic acid, 0.5 mg pyridoxine·HCl, 0.1 mg thiamine·HCl and 30 g sucrose, was used as the standard culture medium. Bacteriological agar (0.6%) was added and the pH adjusted to ~5.7 using a few drops of 1N HCl or 1N NaOH before autoclaving at 105 kPa for 20 min.

Cultures were maintained in the growth room at a constant 25 °C ± 2 °C with a 16 h photoperiod under a light intensity of

Table 1. – Effects of increasing benzylaminopurine levels on axillary shoots proliferation in *Cupressus sempervirens* and *Chamaecyparis lawsoniana*.

BA conc. (mg.l ⁻¹)	Mean No. Shoots per Explant ¹	
	<i>Cupressus sempervirens</i> ²	<i>Chamaecyparis lawsoniana</i> ³
0.0	5.92±0.22 ^d	7.80±0.37 ^g
0.001	7.24±0.19 ^e	9.95±0.71 ^h
0.01	7.93±0.27 ^f	10.10±0.64 ^h
0.1	8.23±0.25 ^f	10.20±0.63 ^h
0.5	ND ⁵	9.90±0.42 ^h
1.0	ND	9.95±0.51 ^h
ANOVA ⁴	***	*

¹) Means followed by the same superscript letter do not differ significantly (P > 0.05; t-test).

²) Data are the means of 76 to 82 replicates per treatment.

³) Data are the means of 20 to 22 replicates per treatment.

⁴) ANOVA: * P < 0.05; ***, P < 0.001.

⁵) ND: not determined.

Table 2. – *In vitro* formation of callus and rooting on shoots of *Cupressus sempervirens* and *Chamaecyparis lawsoniana* on various culture media.

Medium	<i>Cupressus sempervirens</i> ¹		<i>Chamaecyparis lawsoniana</i> ²	
	Callusing (%)	Rooting (%)	Callusing (%)	Rooting (%)
Water agar; 0.1 mg.l ⁻¹ IBA	25.71 (9) ³	28.57 (10)	ND ⁴	ND
½MSM; 1% sucrose	75.00 (18)	50.00 (12)	35.29 (12)	23.53 (8)
½MSM; 1% sucrose; 0.1 mg.l ⁻¹ IBA	94.74 (18)	57.89 (11)	52.00 (13)	40.00 (10)
½MSM; 1% sucrose; 0.5 mg.l ⁻¹ IBA	100.00 (20)	95.00 (19)	78.26 (18)	56.52 (13)
½MSM; 1% sucrose; 1.0 mg.l ⁻¹ IBA	100.00 (20)	85.00 (17)	95.00 (19)	90.00 (18)
½MSM; 2% sucrose; 1.0 mg.l ⁻¹ IBA	100.00 (36)	50.00 (18)	ND	ND
½MSM; 2% sucrose; 0.5 mg.l ⁻¹ IBA	94.44 (34)	36.11 (13)	ND	ND
Chi-square test ⁵	$\chi^2 = 96.69$ $\chi^2 (0.001; df=6)$ = 22.46 ***	$\chi^2 = 35.25$ $\chi^2 (0.001; df=6)$ = 22.46 ***	$\chi^2 = 22.85$ $\chi^2 (0.001; df=3)$ = 16.27 ***	$\chi^2 = 23.60$ $\chi^2 (0.001; df=3)$ = 16.27 ***

¹⁾ 19 to 36 shoots per replicate treatment.

²⁾ 20 to 35 shoots per replicate treatment.

³⁾ Numbers in parentheses are actual recorded values.

⁴⁾ ND: not determined.

⁵⁾ Chi-sq. Test. ***; P < 0.001.

19 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ to 23 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, provided by Phillips MCFE 40W/29 fluorescent tubes.

Shoot multiplication

One step propagation – multiplication was tested. Explants were initially cultured on MSM with 3% sucrose for 10 days for acclimation and elimination of contaminated cultures. For shoot multiplication, contaminant-free explants were transferred to MSM containing 3% sucrose and benzylaminopurine (BA; 0.0 mg.l⁻¹ to 1.0 mg.l⁻¹) for 14 days for axillary shoot proliferation and development. Following, micropropagated shoots (the whole shoot clusters) were transferred to MSM with 2% sucrose, but without growth regulators, for 28 days for conditioning and axillary shoot elongation.

Rooting

After conditioning and shoot elongation, axillary shoots, 20 mm to 30 mm length, were removed from the micropropagated shoots (shoot clusters) and transferred to half-strength MSM (½ MSM) or 0.6% distilled water agar containing 0.0 mg.l⁻¹ to 1.0 mg.l⁻¹ indole butyric acid (IBA), with 0%, 1% or 2% sucrose. Rooting period was 28 days.

Rooting under non-sterile conditions

Following the conditioning – elongation stage, 60 (axillary) shoots of *C. sempervirens* were transferred to 12 glass jars

containing approx. 100 ml of peat-sand (1:1 v/v) moistened with 25 ml sterile distilled water. During transfer, 50% of the shoots were treated with hormone rooting powder (Doff Portland, Nottingham, UK) containing naphthalene acetic acid (NAA) plus Captan. Rooting period was 28 days.

Weaning and aftercare

Rooted shoots were weaned into a loam-peat-sand (7:2:3 v/v/v) compost in 80 mm square pots. Plants were transferred to the misting system in the glasshouse for 14 days at 18 °C–23 °C, before placing under the glasshouse benching for 28 days to complete the hardening-off process. Finally, micropropagated plants were transferred to ambient glasshouse conditions.

Experimental design and statistical analysis

At the shoot multiplication phase, 320 *C. sempervirens* and 130 *Chamaecyparis lawsoniana* contaminant-free explants were available for experimentation.

Treatments were arranged randomly on the shelves in the growth room and the following data recorded: axillary shoot development (number of shoots/explant) after the elongation stage; callus formation and rooting rates (%).

Chi-square (χ^2) test and analysis of variance (ANOVA) were used for statistical analysis of the results. T-test was used for comparison of means from the various treatments.

Table 3. – Callusing and rooting on micropropagated shoots of *Cupressus sempervirens* shoots treated or untreated with naphthalene acetic acid-based rooting powder following transfer to peat – sand (1:1 v/v) compost. Thirty shoots were used per treatment.

Treatment	Callusing (%) ¹	Rooting (%) ²
Peat-sand - rooting powder	16.67 (5) ³	93.33 (28)
Peat-sand + rooting powder	76.67 (23)	96.67 (29)
Chi-square Test ⁴	$\chi^2 = 21.70$ $\chi^2 (0.001; df = 1) = 10.83$ ***	$\chi^2 = 0.35$ $\chi^2 (0.05; df = 1) = 3.84$ n.s.

¹) Occurrence of callusing on shoot base.

²) Percentage shoots forming roots.

³) Numbers in parentheses indicate actual values.

⁴) Chi-sq. test: n.s.; P > 0.05; ***; P < 0.001.

Results

Axillary shoot proliferation

Shoot multiplication was recorded after the elongation stage, before placing in the rooting medium. Large numbers of shoots of both *C. sempervirens* and *Chamaecyparis lawsoniana* were recovered from explants placed on medium lacking BA (Table 1). In comparison to medium lacking BA, however, increasing concentrations of the cytokinin induced significantly higher numbers of shoots in both species (*C. sempervirens*: P < 0.001; *Chamaecyparis lawsoniana*: P < 0.05). With *Chamaecyparis lawsoniana*, increasing the BA concentration above 0.1 mg.l⁻¹ reduced the numbers of shoots recovered compared with media containing 0.01 mg.l⁻¹ or 0.1 mg.l⁻¹ BA.

Rooting

Callus formation and rooting of *C. sempervirens* were markedly different on the 3 basic media tested (Table 2). WA resulted in the formation of significantly less callus than ½ MSM media with either 1% or 2% sucrose (P < 0.001). Also, callusing was greater on media containing IBA than those lacking the growth regulator. Rooting of *C. sempervirens* was greatest on ½ MSM with 1% sucrose and 0.5 mg.l⁻¹ IBA.

With *Chamaecyparis lawsoniana* all rooting media containing IBA induced higher callusing and rooting percentages than found in ½ MSM lacking the growth regulator. Highest rates of root induction were obtained on ½ MSM with 1% sucrose and 1 mg.l⁻¹ IBA.

Rooting under non-sterile conditions

Significantly greater numbers of shoots of *C. sempervirens* treated with NAA rooting powder during transfer to peat-sand compost directly from conditioning produced callus, compared with untreated shoots (P < 0.001; Table 3). No significant differences in rooting rates were found between the two treatments, however, with both treatments resulting in over 90% of shoots forming roots. The ability of *Chamaecyparis lawsoniana* to form roots under these conditions was not tested.

Weaning and aftercare

Rooted shoots of both, *C. sempervirens* and of *Chamaecyparis lawsoniana*, were removed from culture and weaned for 14 days under mist into compost in 80 mm pots. After this period, plants were placed under the glasshouse benching to complete the hardening-off process. Most of the shoots of each species (>80%) survived the weaning process and grew vigorously under glasshouse conditions for over 6 months after transfer from sterile conditions.

Discussion

Six to 8-fold increases in numbers of shoots of *Cupressus sempervirens* and *Chamaecyparis lawsoniana* were recovered from medium lacking BA, with numbers increasing on addition of low levels of this growth regulator. This increase in the number of shoots arises from the indeterminate growth habit of these species, where prolific development of axillary buds occurs on all shoots during normal growing conditions. Addition of cytokinins to the medium is important for the development of axillary buds in other coniferous genera, such as *Pinus* (ABDULLAH *et al.*, 1986; MOTT and AMERSON, 1982) and *Picea* (KUNZE *et al.*, 1993), and BA has previously been reported to stimulate shoot production in *Cupressus species* (THOMAS *et al.*, 1977; FOSSI *et al.*, 1981; HRIB and DOBRY, 1984; FRANCO and SCHWARZ, 1985). Several studies, however, have demonstrated that axillary bud development can occur in the absence of added cytokinins in certain coniferous species (JOHN and WEBB, 1987; WOODWARD, 1987; ZOGLAUER *et al.*, 1992). Addition of cytokinins to multiplication media can induce hyperhydricity (PAQUES, 1991) and can increase the levels of somaclonal variation, although such variation may prove useful in obtaining further *Cupressus* clones showing resistance to *Seiridium* canker (HUANG *et al.*, 1993). The ability to produce micropropagated shoots of *Cupressus* clones with known resistance to canker without application of cytokinins could prove beneficial in clonal forestry programmes.

In commercial production of plants by micropropagation, a multiplication rate of approximately 2.5fold to 3.5fold per monthly sub-culture is considered optimum for handling purposes in large laboratories (CONSTANTINE, 1987). The multiplication obtained in this work on *C. sempervirens* and *Chamaecyparis lawsoniana* exceeded these figures, but the rates were within manageable limits.

Rooting of both species occurred on ½ MSM in the absence of added auxin, although rooting efficiency was greatly increased on addition of IBA. Reduction of sucrose concentrations in media from 2% to 1% increased the numbers of shoots forming roots in *C. sempervirens*. These levels of rooting are an improvement upon the 80% previously reported for *C. sempervirens* (FOSSI *et al.*, 1981) and obtained by dipping micropropagated shoots into a solution containing 1 mg.l⁻¹ naphthalene acetic acid. Addition of indole acetic acid to media was necessary to stimulate rooting in *C. dupreziana* (HRIB and DOBRY, 1984). In many other coniferous genera, the presence of auxin in rooting media is a pre-requisite for root initiation (MOTT and AMERSON, 1982; RANCILLAC *et al.*, 1982; RUMARY and THORPE, 1984; ZEL *et al.*, 1988).

Callusing occurred on all media used, although amounts increased with increasing concentrations of IBA. The survival of *in vitro*-rooted shoots following weaning, however, indicates that the callus did not interfere with vascular connections within the plants.

Under non-sterile conditions over 90% of *C. sempervirens* shoots rooted, regardless of the use of NAA-based rooting powder. FRANCO and SCHWARZ (1985) reported *ex vitro* rooting of *C. lusitanica*, although repeated application of a solution containing 2.8 mg.l⁻¹ NAA was necessary to induce 30% rooting in this species. The use of an *ex vitro* rooting step greatly improves the economic efficiency of micropropagation (CONSTANTINE, 1987), and increases the likelihood of such a system proving viable for *Cupressus* spp. and *Chamaecyparis* spp..

Multiplication (6fold to 10fold), rooting (up to 95%) and weaning survival (>90%) rates obtained with *C. sempervirens* and *Chamaecyparis lawsoniana* were acceptable for commercial production. *C. sempervirens* also rooted in compost and survived weaning in acceptable numbers. The methods described in this paper provide a sound basis for the development of efficient techniques for the production of *Cupressus* species using micropropagation, and for the rapid increase of genotypes showing resistance to *Seiridium* canker in screening programmes (SPANOS, 1995).

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Genetic Variation Across the Natural Distribution of the South East Asian Pine, *Pinus kesiya* ROYLE ex GORDON (Pinaceae)

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Summary

Allozyme variation has been assessed in 9 populations (172 families) of the economically important south east Asian pine,

Pinus kesiya ROYLE ex GORDON, from across its entire geographical range, using 12 isozyme loci identified by 7 enzyme systems. The mean percentage of polymorphic loci per population, the mean number of alleles per locus and the mean genetic diversity within populations were 45.4%, 1.6 and 0.153 respectively. Genetic diversity was greatest in populations from the south-eastern populations and lowest in north-western populations. In the present study G_{ST} (0.121) was high compar-

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