PROPAGATION OF RHODODENDRONS BY-TISSUE CULTURE: PART 1. DEVELOPMENT OF A CULTURE MEDIUM FOR MULTIPLICATION OF SHOOTS[1 2]

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Abstract. It was necessary to reduce the potassium concentration in the Murashige and Skoog (MS) formula (3) for sustained shoot growth of rhododendron seedlings and shoots. The KNO_3 concentration was reduced to 950 mg/l and the NH_4NO_3 increased to 2000 mg/l. Other constituents required in the basal medium for the Stage II shoot multiplication phase were: adenine sulfate dihydrate 80 mg/l; IAA 4 mg/l; and N^6 (Δ^2-isopentenyl)-adenine (2iP) 15 mg/l. This medium supported the development of 6.2 new shoots per culture in 8 weeks. The new shoots arose from axillary buds of the original shoot explants and axillary buds of new developing shoots.

INTRODUCTION

Three distinct stages were described by Murashige (1) for rapid plant propagation through tissue culture. Murashige, et al (2) developed the basic principles for these three stages with their research on gerbera propagation that is now a commercially accepted procedure. Stage I involves establishing an aseptic tissue culture from shoot tips or other plant organs. During Stage II, conditions are altered to cause a rapid increase of shoots or other organs that will ultimately produce complete plants. Stage III involves changing cultural conditions to promote root development on the shoots and to harden these plants in preparation for successful transfer to soil.

The development of a tissue culture method for vegetatively propagating rhododendrons has commercial value in reducing the time required to increase stock plants of new clones. It is estimated 10 years are required to develop sufficient stock of a new clone for large volume sales. A technique for rapidly producing stock plants in the initial period could shorten development by several years.

The tissue culture method also has potential in propagating clones that are hard to root by the normal cuttage methods.

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Preliminary experiments on establishing rhododendron tissue cultures showed that the MS formula (3) contained something toxic. Tissue in contact with the medium rapidly turned brown and browning progressed up the seedling stem until the cultures were completely killed. Sub-culturing the green portion of the shoot on a 3 week schedule kept them alive. The browning problem was eliminated by reducing the nitrogen-containing compounds in the MS formula to one-half.

This paper is concerned with the establishment of Stage II culture conditions for rapid multiplication of rhododendron shoots.

MATERIALS AND METHODS

Plant materials were obtained from aseptically-grown rhododendron seedlings and from shoot tips of 'Rose Elf' plants. Seed was collected and stored in the following manner in order to avoid severe culture contamination. Green capsules were collected and surface sterilized either for 1-2 minutes in 70% ethanol or for 20 minutes in a 10% water solution of laundry bleach, with a small amount of Tween 20 (polyoxyethylene sorbitan) added. The capsules were then air dried. Seeds were hand shaken out of the dehisced capsules with care not to cause capsule fragmentation since small pieces of this tissue were a major source of contamination. Before germinating, 20 mg lots of seeds were surface sterilized for 20 minutes in a water solution of 10% laundry bleach containing 0.1% Tween 20. The surface sterilization reaction was stopped by decanting off the sterilant and rinsing the seeds several times in sterile water. The seeds were suspended in 5 ml of sterile 0.2% water-agar. The seed suspension was poured into 16 oz. French squares or 125 ml Erlenmeyer flasks containing 50 ml of basal medium. The containers were then swirled to spread the seed evenly over the medium surface. Seedlings grew to sufficient size for further culturing or transplanting in 2-3 months.

'Rose Elf' shoot-tip explants were prepared by removing the expanding leaves and washing the shoot tips in soapy water. They were then soaked for 2-3 hours in an antioxidant solution consisting of 150 mg citric acid and 100 mg ascorbic acid diluted to 1 liter with water. Shoot tips were surface sterilized for 15 minutes in 10% laundry bleach water solution containing 0.1% Tween 20, then were rinsed several times with sterile water. The shoot tips were then aseptically placed on the medium. The explants started a new flush of growth after 6-9 weeks in culture.

The basal nutrient medium consisted of the following constituents and concentrations:

Constituent	Concentration mg/l
Inorganic salts	Modified Murashige and Skoog formula (low K)
Additional phosphate as $NaH_2PO_4 \bullet H_2O$	170
Sucrose	30,000
Difco Bacto-agar	10,000
i-inositol	100
thiamine HCl	0.4
IAA	0.25

Organic compounds, including the auxins and cytokinins, were dissolved in 1-2 ml dimethyl sulfoxide before dilution with water. Aliquotes of these stock solutions were added to the medium before autoclaving. The medium was adjusted to pH 4.5 by adding in NaOH or HCl before autoclaving. The medium was dispersed at 25 ml per 25 x 150 mm culture tubes and autoclaved 15 minutes at 121°C. Polypropopylene tube closures (Bellco Kaputs) were used to close the tubes. After autoclaving, the tubes were routinely cooled with the medium solidified on a 45° angle.

Cultures were kept in a room with temperatures that ranged between 19° and 23°C. Lighting was by cool white fluorescent lamps with an intensity of ca. 1000 lux on the cultures, and with a daylength of 16 hours.

RESULTS

Varying the concentrations of NH_4 and NO_3 concentrations in the MS formula (3) had no effect on the toxic tissue browning condition in shoots. However, a large proportion of the total N concentration in the formula is KNO_3. When it was reduced, the browning and early culture death symptoms were eliminated. The KNO_3 treatment with maximum growth was 950 mg/l (table 1), one-half the concentration in the MS formula. The total N concentration of the modified medium remained the same as the MS formula as an equivalent amount of N was added as NH_4NO_3.

An addition of 170 mg/l $NaH_2PO_4 \bullet H_2O$ was found to be beneficial for growth of all rhododendron cultures. Adenine sulfate dihydrate at 80 mg/l was important for axillary bud breaking and sustained shoot growth in Stage II.

Critical for the best Stage II culture conditions is the determination of optimum combinations and concentrations of auxins and cytokinins. Initial experiments indicated that indole-3-acetic acid (IAA) gave growth superior to that from β-naphthaleneacetic acid (NAA). Normal growth of shoots occurred at 0.5 to 5.0 mg/l IAA. Comparing kinetin and N_6-benzyladenine (BA) on bud differentiation showed that BA was slightly more effective than kinetin at concentrations of 2.5-5.0 mg/l (table 2). BA generally seemed to be quite toxic to rhododendron tissue.

The most effective concentrations for developing the maximum number of shoots were 4 mg/l IAA and 15 mg/l N^6-(Δ^2 isopentenyl)-adenine (table 3). At this optimum concentration 4.1 shoots were obtained in 5 weeks and 6.2 shoots in 8 weeks. Shoot development on the explant reached a maximum in 7 weeks as the shoots showed some senescence and deterioration in 8 weeks.

Shoot tips from 'Rose Elf' rhododendron were established in culture and tested on the Stage II medium. After five weeks an average of 3.2 shoots per explant was obtained.

Figure 1.

(a) Rhododendrons developed from seeds after 3.5 months in culture.

(b) Shoots developed from the seedling explant cultured for 5 weeks on medium containing 2iP.

(c) Shoot development after 8 weeks of culture as affected by IAA (4 mg/l) plus various concentrations of 2iP. Left to right: 0, 5, 10, 15 mg/l 2iP.

(d) Seedling plant developed from tissue culture 3.5 months after transplanting in soil.

(e) Rose Elf shoot apex culture 6 weeks after explanting.

(f) Rose Elf shoots 6 weeks after subculturing on the basal rhododendron medium.

Table 1. Effect of varying K concentration with total N remaining constant on the survival rate of rhododendron seedling explants and shoot growth after 6 weeks of culture.

Medium Composition KNO_3 mg/l	NH_4NO_3 mg/l[2]	Survival %	Fresh wt[1]/ mg
0	2400	100	6.6±0.8[2]
475	2200	100	9.0±1.8
950	2000	100	9.6±1.5
1425	1800	100	6.4±1.1
1900[3]	1600[3]	70	4.7±1.0

1/ 10 replicate cultures per treatment. Fresh wt. of explants was 4.4±0.6 mg.

2/ Standard error of the mean

3/ Concentration of KNO_3 and NH_4NO_3 in MS formula

Table 2. Effects of kinetin and BA concentrations on the survival rate of rhododendron explants and the numbers of shoots after 7 weeks in culture. The IAA concentration was 1 mg/l.

Cytokinin	mg/l	Survival %	No. of Shoots/ Culture
	0	90	1.3±0.1[2]
Kinetin	2.5	60	1.0±0.0
Kinetin	5.0	70	1.6±0.3
Kinetin	10.0	60	1.5±0.5
Kinetin	20.0	70	2.7±0.7
BA[1]	2.5	30	3.0±0.6
BA	5.0	60	2.0±0.3
BA	10.0	50	1.4±0.2
BA	20.0	30	3.0±1.2

1/ N_6 - benzyladenine

2/ Standard error of the mean

Table 3. Effect of IAA and 2iP concentrations on the number of rhododendron shoots arising per explant.

IAA mg/l	after 5 weeks 2iP mg/liter				after 8 weeks 2iP mg/liter			
	0	5	10	15	0	5	10	15
0	1.2±0.1[1]	2.8±0.3	2.8±0.2	3.0±0.2	1.6±0.2	4.4±0.4	4.0±0.7	4.2±0.7
2	1.0±0	1.8±0.4	2.6±0.4	2.6±0.3	1.6±0.2	2.4±0.7	2.6±0.4	4.2±0.6
4	1.6±0.3	2.7±0.4	2.8±0.3	4.1±0.4	2.0±0.4	3.0±0.3	3.6±0.6	6.2±1.0
6	1.1±0.1	2.6±0.2	2.5±0.3	2.2±0.3	1.0±0	3.2±0.6	4.4±0.2	3.8±0.7

1/ Standard error of the mean

Table 4. The composition of Stage II rhododendron medium.

Mineral Salts	mg per liter
NH_4NO_3	2,000
KNO_3	950
$CaCl_2 \bullet 2H_2O$	440
$MgSO_4 \bullet 7H_2O$	370
KH_2PO_4	170
$NaH_2PO_4 \bullet H_2O$	170

Table 4. continued

Na_2EDTA	37.3
$FeSO_4 \bullet 7H_2O$	27.8
$MnSO_4 \bullet H_2O$	16.9
$ZnSO_4 \bullet 7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MoO_4 \bullet 2H_2O$	0.25
$CuSO_4 \bullet 5H_2O$	0.025
$CoCl_2 \bullet .6H_2O$	0.025
Organic Constituents	**mg per liter**
Sucrose	30,000
Bactoagar	8,000
i-inositol	100
adenine sulfate.dihydrate	80
2iP (N^6-(Δ^2-isopentenyl)-adenine)	15
IAA (Indole-3-acetic acid)	4
Thiamine. HCl	0.4

DISCUSSION

The potassium concentration in the MS formula was toxic to rhododendron tissue and a reduction of the KNO_3 concentration by one-half eliminated tissue browning. Pierik and Steegmans (4) reported ½ strength Knops solution was best for rooting of rhododendron stems and attributed the lower salt concentration in *Knop's formula to be the major factor. The MS* medium has a 16 fold greater salt concentration based on millimoles (mM) /l (3,5). The K concentration of ½ strength Knops formula is 1.1 mM/l compared to 10 mM/l in the modified rhododendron formula. Possibly the high salt concentrations in the MS medium have a buffering effect that reduced the toxicity of K on rhododendrons.

The essential compounds required for shoot development in the basal medium were 80 mg/l of adenine sulfate dehydrate, 15 mg/l of 2iP and 4 mg/l of IAA. This combination of compounds resulted in an average increase of 6.2 shoots per culture in 8 weeks.

The cytokinin 2iP was the preferable chemical as it caused the greatest number of axillary buds to develop into shoots without any toxicity effects.

Rapid rhododendron shoot multiplication arising from axillary buds has been demonstrated. However, parameters for Stages I and III remain to be thoroughly investigated.

LITERATURE CITED

1. Murashige, T. 1974. Plant propagation through tissue cultures. *Ann. Rev. Plant Physiol.* 25:135-166.

2. ——————, M. Serpa and J. B. Jones. 1974. Clonal multiplication of gerbera through tissue culture. *HortScience* 9:175-180.

3. ——————— and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.

4. Pierik, R. L. M. and H. H. M. Steegmans. 1975. Analysis of adventitious root formation in isolated stem explants of Rhododendron. *Sci. Hort.* 3:1-20.

5. White P. R. 1963. The Cultivation of Animal and Plant Cells. The Ronald Press Co. p. 59.