Use of Micropropagation for the Conservation of Rare Cornish Garden Plants at Risk from *Phytophthora ramorum* ©

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INTRODUCTION

The historic gardens of Cornwall contain a unique heritage of plant species introduced from the mid 1800s onwards by plant hunters such as Sir Joseph Dalton Hooker and Frank Kingdon Ward. Cornwall's mild climate was ideal, enabling plants such as rhododendrons, magnolias, and camellias to be grown outside and these have now become the mainstay of Cornish gardens. Recently these historically important introductions and subsequent hybrids have become threatened by the fungal diseases *Phytophthora ramorum* and *P. kernoviae.* As these diseases are currently regarded as non-endemic any infected material has to be destroyed (Defra, 2008). The Rare Species Laboratory of Duchy College has responded to the phytophthora problem by proposing to clone rare plants at risk from infection to prevent permanent loss of this historically and botanically important material. The plants initially most at risk from infection were rhododendrons.

Conventional propagation methods for rhododendrons were unsuitable due to the age of the specimens. Low rooting success from cuttings was anticipated (Gardiner, 2002), furthermore infected propagules might transfer the disease on reinstatement to the garden environment. A method of propagation in sterile culture was therefore required that could be used on older plants and which would facilitate early detection of the presence of *Phytophthora* species. Micropropagation appeared to be a sensible approach.

Different parts of rhododendron plants have been used for shoot regeneration in sterile culture ranging from shoot tips (Anderson, 1975) to floral tissues such as pedicels and ovary bases (Meyer, 1982), ovaries (Dai et al., 1987), stamens (Shevade and Preece, 1993), and more recently ovary and pedicel (Tomsone and Gertnere, 2003). The floral bud, being enclosed in a tight covering of leaf sheaths, provides a relatively sterile explant compared with exposed shoots.

This paper reports on a range of micropropagation techniques that were developed from Spring 2005 to successfully conserve important rhododendron germplasm and produce disease-free rooted plantlets for eventual reintroduction. To date, material from 16 Cornish gardens and five gardens outside Cornwall has been processed, totalling more than 400 accessions.

MATERIALS AND METHODS

Material for micropropagation had to be taken from plants growing in situ in Cornish historic gardens. For each accession between three and six stems were collected, usually by the owners or head gardeners in order to select the most important cultivars. Great care was taken in the handling, movement, and storage of these

samples between garden and laboratory, and particularly in the disposal of waste material throughout processing. The techniques are described in the order in which they were tried.

Technique 1: Vegetative Shoots. These were taken at various times of year according to the urgency of propagation.

Initially, young vegetative buds were chosen in situ just before bud burst in April/May and the older wood was sprayed with a solution of 0.1% sodium hypochlorite. Each shoot was then enclosed within a perforated flower sleeve to give some protection to the developing bud without encouraging high humidity and possible increased fungal activity.

After bud burst, the juvenile growth, together with approximately 10 cm of older stem, was removed from the plant, sealed in a polythene bag and brought to the laboratory. Here the older leaves were reduced in number and length and the old wood disinfected by scrubbing with a solution of approx. 0.3% sodium hypochlorite, followed by rinsing off and drying with tissues. Stems were then shortened and placed in flower food solution and in natural light for 2–3 weeks to develop further. The young growth of 2 to 3 cm length was then removed and washed in distilled water containing a few drops of surfactant for 15 min and transferred to a sterilizing solution of 0.2% sodium hypochlorite and agitated for 20 min. After thorough rinsing in sterile distilled water, shoots were re-cut and placed in sterile containers of Anderson's rhododendron media (Anderson, 1978) containing IAA (indole-3-acetic acid) at 1 $mg \cdot L^{-1}$; 2iP (N -[2-isopentenyl]adenine) at 5 $mg \cdot L^{-1}$; sucrose at 30 $g \cdot L^{-1}$; and agar and the pH was adjusted to 5.0 prior to autoclaving. All explants in the research programme were maintained at a temperature of 22 to 23 °C under fluorescent tubes (16-h duration/day).

We also used the technique for young shoots taken from other plants in July, at a more mature stage of growth. These were deleafed, trimmed to 3 cm length, washed under running water for 30 min, and sterilized in a stronger solution of 0.25% sodium hypochlorite for 20 min.

It was also necessary to work with shoots from three "condemned" rhododendrons in the autumn (September). In this case a higher concentration of sodium hypochlorite (0.3%) was used as a sterilant as it was thought that the matured stems could withstand this concentration.

Technique 2: Floral Buds. Buds were collected from a number of gardens between February and April and consisted of a range of developmental stages from very tight bud up to partial bud burst. The accessions were picked on only one date from each garden, hence there was variability in the stage of development. Floral buds were collected with 10 to 15 cm of stem, the foliage was cut off leaving the petioles and the samples were securely bagged as described above for the vegetative shoots technique. On receipt, samples were cold stored at 4 °C for up to 2 weeks until processed.

To prepare the floral bud for sterilization, outer leaf scales were carefully prised off until nearing the florets. The whole floral bud was then scrubbed with a solution of 0.5% sodium hypochlorite together with bacterial hand wash before removal from the stem, which was replaced in the cold store for possible future use. The bud surface was sterilized in 0.3% sodium hypochlorite for 20 min before rinsing three times with sterile distilled water. The bud was carefully dissected and individual

florets were excised with as much pedicel as possible. These were soaked for 15 min in an antioxidant solution (200 mg·L^{-1} ascorbic acid and 150 mg·L^{-1} citric acid) together with 1 mg·L^{-1} thidiazuron. The individual florets were then placed either vertically or slanting with the pedicel in Anderson's medium (Anderson 1984) containing 30 g·L^{-1} sucrose and supplemented with 1.9 mg·L^{-1} IAA; 5 mg·L^{-1} 2iP, and 1 mg·L^{-1} thidiazuron. The pH was adjusted to 5.5 prior to autoclaving. Each container supported four or five florets, according to their size.

Technique 3: Lateral Vegetative Buds. This technique was used when floral tissues proved not to have been decontaminated successfully. The excised stems stored from Technique 2 were examined 2–3 months later. Where lateral buds were well developed these were treated as per the floral buds, with a layer of outer scale leaves being removed before sterilization and a further layer removed before soaking. Buds were then placed in Anderson's media (Anderson, 1978) together with the plant growth regulators used in Technique 2.

Technique 4: Terminal Vegetative Buds. On rhododendrons where there were no floral buds to use, vegetative buds had been collected. These had been cold stored for 2 months and were treated as in Technique 3 and placed in the same media formulation.

RESULTS

A successful result for all techniques was indicated by both the absence of fungal, bacterial, or algal contamination of the nutrient gel and the initiation of shoots. If contamination was present it would be visible within a 3-week period for Technique 1, but might take up to 2 months to appear in the other techniques due to the slow expansion of the petals/scale leaves.

Table 1 shows that of all the techniques used, floral buds have proved to be the most reliable for creating clean cultures. Out of the 380 floral bud accessions processed, 65% have been successfully micropropagated to date. This technique has also provided more rapid proliferation than any of the protocols using vegetative tissue as can be seen in Fig.1, where each floret within the floral bud (there may be up to 20 florets per bud) could produce 20 or more shoots.

Despite the low decontamination rate of the initial vegetative treatment, the four clean cultures have proliferated well and the progeny were returned to the relevant garden owners.

Table1. Success rates of sterilization techniques on *Rhododendron* tissues.

Technique	Description	Total treated	Successful sterile cultures	Success (%)
1	Bud burst/April-May	25	4	16
	July	25	5	20
	September	3	0	0
2	Floral buds	380	247	65
3	Lateral buds	37	5	13
4	Terminal buds	19	2	10

Technique 1, using young shoots, provided disappointing results despite all the mother plant preparation. This could be due to the high populations of contaminants around the matured bark. Tissues exposed to contamination for longer periods throughout the year would be expected to be more difficult to decontaminate, as is shown by the results. The use of the matured vegetative bud (Techniques 3 and 4) has, where successful, rapidly produced multiple shoots (Fig. 2).

Figure 1. Shoot regeneration from florets.

Figure 2. Shoot regeneration from lateral vegetative buds.

DISCUSSION

The major obstacle to the successful micropropagation of ancient rhododendrons has been one of overcoming external contaminants. Many of the accessions were covered by hairs or a dense indumentum, or were extremely sticky and visibly trapped contaminants. Wet weather at the time of collection also influenced the outcome, as it was observed that wet material was less likely to be decontaminated successfully than dry material. Micropropagation from floral tissue is the preferred technique for successful regeneration provided that the bud is collected before the outer scale leaves have started to loosen and admit contaminants.

The methodology used for each accession during this conservation programme has been of a progressive nature. Four accessions were successfully propagated from the initial technique, but others may have had to progress through all techniques before decontamination was successful. To check that material is disease free following micropropagation, plants are tested for the presence of *P. ramorum* and *P. kernoviae* by using the Lateral Flow Device approved by the Department for Environment, Food, and Rural Affairs.

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