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Propagation Methods for Environmental Offset Planting of the Kogan Waxflower (*Philotheca sporadica*) (Rutaceae)

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ABSTRACT

The Kogan waxflower (*Philotheca sporadica*) is a threatened and geographically restricted plant species that is being impacted by gas pipeline construction. This study assessed seed, cutting and tissue culture methods for propagation of *P. sporadica* for an environmental offset program. Fresh seed could not be germinated under nursery conditions and less than 1.5% of cuttings produced roots. However, *in vitro* proliferation of shoots in tissue culture media containing 4.4 μM benzyladenine provided over 5,000 shoots from each initiated shoot tip in a 6 month period. Shoots were converted readily into plantlets using 314.9 μM indole-3-butyric acid for root induction and an *in vitro* soil-less system for transferring plantlets from the laboratory to the nursery. These results demonstrate that offset planting programs can be hampered by low amenability of plant species to propagation but that identifying a successful propagation method can lead to rapid gains in plant production.

Key words: Conservation, cuttings, *Eriostemon*, seed, tissue culture, translocation

INTRODUCTION

Many threatened plant species occur outside protected reserves, leaving them vulnerable to the impacts of human activities such as agriculture and mining. Pipelines for coal seam gas in southern Queensland, Australia, often traverse agricultural properties that contain significant tracts of native vegetation. Impacts upon threatened plants in this region typically require the establishment of newly propagated populations to offset the impacts of pipeline construction. Critical to the success of these environmental offset plantings is an understanding of possible propagation methods for the impacted species.

One of the plant species impacted by gas pipeline construction is the Kogan waxflower (*Philotheca sporadica*) (formerly *Eriostemon sporadicus*) which is a small and horticulturally attractive shrub that is listed as 'Vulnerable' under the Australian Environment Protection and Biodiversity Conservation (EPBC) Act (1999). This species is confined within a 50×50 km area in southern Queensland (Halford, 1995), with very few plants protected in reserves. Two of the largest populations of the species are being impacted by pipeline construction and so a restoration program has commenced with the aim of propagating *P. sporadica* plants for planting at offset sites.

Offset planting of *P. sporadica* could be limited by low amenability to propagation, as *Philotheca* and *Eriostemon* species are considered difficult to propagate by seeds and cuttings (Nixon, 1980; Ellyard, 1984; Ault, 1994; Lidbetter *et al*., 2003). Low amenability to traditional

propagation can sometimes be overcome using tissue culture which can potentially produce very large numbers of plants from a limited source of plant material (George, 1993; Trueman *et al.*, 2007). This study assessed the amenability to propagation of *P. sporadica*, determining which propagation methods (seed germination, cutting propagation or tissue culture) were suitable for plant production. The results will help to guide offset plantings of threatened plant species and to develop propagation methods for other horticulturally attractive species of Rutaceae.

MATERIALS AND METHODS

Fruit and shoots of *P. sporadica* were collected in June, August, November and December 2011 from natural populations in the vicinity of Kogan (27°02'S, 150°46'E), Queensland, Australia. Approximately 1-2 g of fruit per plant (when available) was collected from 60 plants of each of the two impacted populations. Fruit were stored in sealed paper bags. Shoots were harvested from the same 120 plants and supplemented with shoots from plants in ten neighbouring populations. Shoots were placed in plastic clip-lock bags with a light spray of water, kept cool in tubs containing ice-bricks and transported overnight to the University of the Sunshine Coast (USC) (26°43'S, 153°04'E).

Fruit were allowed to dry and open under ambient laboratory conditions and the seeds from each plant were transferred to Petri dishes. A sample of 20-100 seeds per plant was sown in February 2012 in the seedling potting mix described by Trueman *et al.* (2013a, b). Seeds were covered with a thin layer of vermiculite and the seedling trays were placed under light-mist irrigation (1 min every 5 min) in a translucent-white polyethylene chamber at USC. Germination was monitored until June 2012.

The shoots from each plant were dissected into multi-nodal cuttings, each approximately 5 cm in length, upon arrival at USC. Each cutting was dipped 0.5 cm into powder containing 8 g kg^{-1} Indole-3-Butyric Acid (IBA) for 1 sec and placed 1 cm deep into a 70 mL propagation tube containing cutting mix. The cutting mix consisted of a 75/25 (v/v) mixture of perlite and shredded pine bark with added nutrients, as described by Trueman *et al.* (2013a, b). The cuttings were placed under light-mist irrigation (1 min every 15 min) in a translucent-white polyethylene chamber. All cuttings were assessed for root formation until May 2012.

Excess shoot material from the second and third collections (August and November 2011) was used to initiate tissue cultures. Batches of approximately 30 shoot tips, each 2-3 mm in length and with the macroscopic leaves removed, were washed in 70% ethanol (v/v) for 1 min in 70 mL vials containing one drop of Tween 20. They were then rinsed in sterile distilled water for 1 min and transferred into new vials containing 5% sodium hypochlorite with one drop of Tween 20. The vials were swirled for 30 min on an orbital shaker at 110 rpm and the shoot tips were then rinsed three times in sterile distilled water. Shoot tips were placed on sterile paper to remove excess liquid between solutions. Shoot tips were plated (five tips per 90-mm Petri dish) onto shoot induction medium consisting of half-strength Murashige and Skoog (MS) medium with 30 g L^{-1} sucrose, solidified with 8 g L^{-1} agar and with pH adjusted to 5.8 prior to autoclaving (121°C, 20 min). The shoot tips were maintained at 25°C for 4 weeks under a 16 h photoperiod (~50 μmol m^{-2} sec^{-1} with fluorescent tubes).

Uncontaminated, growing shoot tips were then transferred to 375 mL glass jars containing 50 mL of shoot proliferation medium (Hung and Trueman, 2012) consisting of full-strength MS medium with 30 g L^{-1} sucrose and 4.4 μM benzyladenine (BA), solidified with 8 g L^{-1} agar and with pH adjusted to 5.8 prior to autoclaving (121°C, 20 min). Shoots were proliferated in this medium

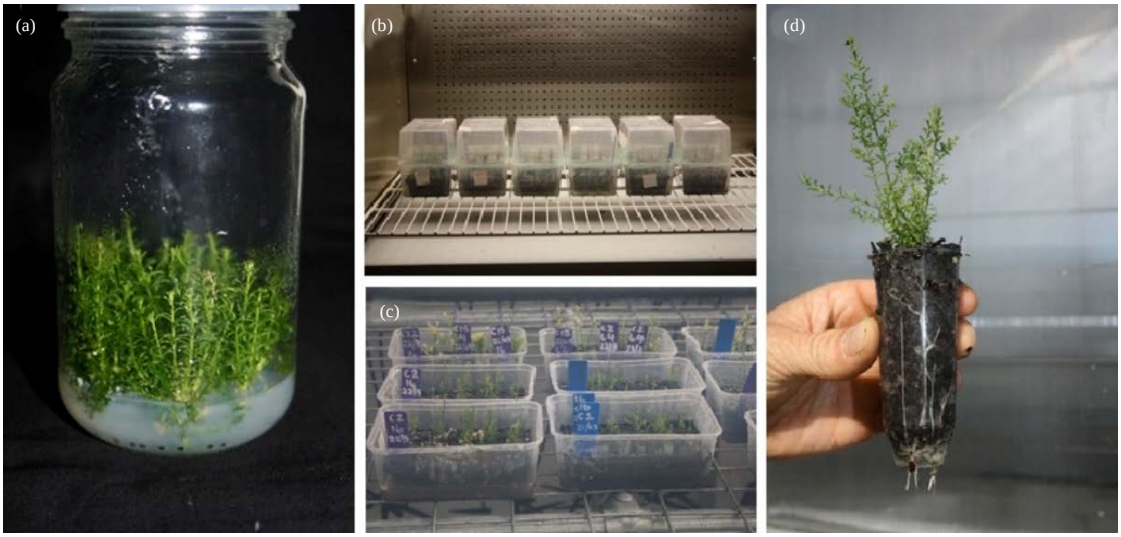

Fig. 1(a-d): Shoot tip culture and plantlet production of *Philotheca sporadica* (a) Shoot proliferation in medium containing 4.4 μM benzyladenine, (b) Plantlets in sealed punnets containing sterilized potting mixture, (c) Plantlets in unsealed punnets in the glasshouse and (d) Plantlet in a 70 mL tube

for 4 week passages at 25°C under a 16 h photoperiod (~100 μmol m^{-2} sec^{-1}) (Fig. 1a). Most shoots were then transferred to shoot elongation medium consisting of hormone-free full-strength MS medium with 30 g L^{-1} sucrose, solidified with 8 g L^{-1} agar and with pH adjusted to 5.8 prior to autoclaving (121°C, 20 min). Shoots were maintained in this medium at 25°C under a 16 h photoperiod (~100 μmol m^{-2} sec^{-1}).

A subsample of shoots of at least 25 mm length was selected for root induction at the end of the shoot proliferation period. These shoots were dissected and transferred randomly to root induction medium consisting of half-strength MS medium with 30 g L^{-1} sucrose and various concentrations of IBA (0, 4.9, 19.7, 78.7 or 314.9 μM), solidified with 8 g L^{-1} agar and with pH adjusted to 5.8 prior to autoclaving (121°C, 20 min). This experiment contained 66-71 replicate jars per treatment, with 10 shoots in each jar. The shoots were placed in darkness for 1 week at 25°C to allow formation of root primordia. Shoots were then transferred to hormone-free half MS medium with 30 g L^{-1} sucrose, solidified with 8 g L^{-1} agar and with pH adjusted to 5.8 prior to autoclaving (121°C, 20 min). Conversion into plantlets occurred at 25°C for 3 weeks under a 16 h photoperiod (~100 μmol m^{-2} sec^{-1}).

Another subsample of shoots was selected for root induction after one 4 week passage on shoot elongation medium. These shoots were treated in the same way as the previous subsample except that only the three highest IBA concentrations (19.7, 78.7 and 314.9 μM) were tested. This experiment contained 62-67 replicate jars per treatment, with 10 shoots per jar. Rooting data was analysed by ANOVA and Tukey's HSD test, with differences regarded as significant at $p<0.05$. The same method for conversion to plantlets was used, subsequently, for all shoots but using only the highest IBA concentration (314.9 μM) for root induction.

The plantlets were transplanted into punnets containing sterile seedling mix, based on the *in vitro* soil-less (IVS) method of Newell *et al.* (2003, 2005) and Hung and Trueman (2012). The

punnets, each containing fifteen 12 mL tubes, were placed in sterile 1 L plastic containers. The containers were watered with sterile distilled water and the container lid was replaced with another container and sealed with plastic film to create a volume of 2 L (Fig. 1b). The sealed punnets were exposed to four different light intensities (~100, 150, 230 and 280 μmol m^{-2} sec^{-1}) over 3 weeks to prepare the plantlets for the nursery environment. The punnets were then moved to a glasshouse and received watering for 30 min, 4 times a day. The lids were removed from the punnets after 4 days to commence hardening-off (Fig. 1c). The plants were re-potted after 3 weeks into 70 mL propagation tubes containing non-sterile seedling mix (Fig. 1d). Plant survival was recorded in June 2012.

RESULTS AND DISCUSSION

Flowers were present on *P. sporadica* in June and August and seeds were obtained from fruits, collected in November and December but no seeds germinated in the nursery. The seeds of many Rutaceae species, including *Philotheca* and *Eriostemon*, are difficult to germinate (Nixon, 1980; Lidbetter *et al*., 2003; Merritt *et al*., 2007; Koch *et al*., 2009). Low seed germination can be the result of physical, physiological or morphological dormancy mechanisms that allow the accumulation of a persistent soil seed bank and prevent germination before suitable conditions for seedling establishment (Baskin and Baskin, 2004; Finch-Savage and Leubner-Metzger, 2006). Seed dormancy mechanisms in Rutaceae species are poorly understood although long periods of seed burial or smoke treatments are prerequisites for germination of some species including *Eriostemon spicatus* (Dixon *et al*., 1995; Bell, 1999; Merritt *et al*., 2007; Turner and Merritt, 2009). Seed of *P. sporadica* could not be germinated without scarification, stratification or other dormancy-breaking methods and so seedling progeny from the impacted population did not contribute to the plants produced for the offset population.

Propagation of *P. sporadica* cuttings was very difficult, with only 138 cuttings (from 63 individuals) out of 17,742 cuttings forming roots (Table 1). Species from the sister genus, *Eriostemon*, are also difficult to propagate from cuttings (Ellyard, 1984; Ault, 1994; Lidbetter *et al*., 2003). The ability to propagate cuttings can be highly seasonal, with greater success often obtained in spring or summer than autumn or winter (Leakey, 2004; Trueman *et al*., 2013a, b). Rooting of *P. sporadica* cuttings was higher in spring and summer than in winter but no season provided greater than 1.4% rooting (Table 1).

Tissue culture was, by far, the most effective method for producing *P. sporadica* plants. Shoot tip culture provided over 75,000 shoots (from 13 individuals) in about 6 months from culture initiation. Root formation was greatest using the highest concentration (314.9 μM) of IBA (Fig. 2). This IBA concentration provided higher rooting when the shoots had been maintained for 4 weeks on BA-free elongation medium (55%) than immediately after the shoots were on BA-containing proliferation medium (34%). The plantlets grew vigorously and acclimatized readily to nursery

Table 1: Number and percentage of *Philotheca sporadica* cuttings that formed roots in four different collection periods

Collection period	No. of cuttings	Cuttings with roots	Cuttings with roots (%)
June	6,658	8	0.1
August	2,029	8	0.4
November	4,672	64	1.4
December	4,383	58	1.3
Total (mean)	17,742	138	(0.8)

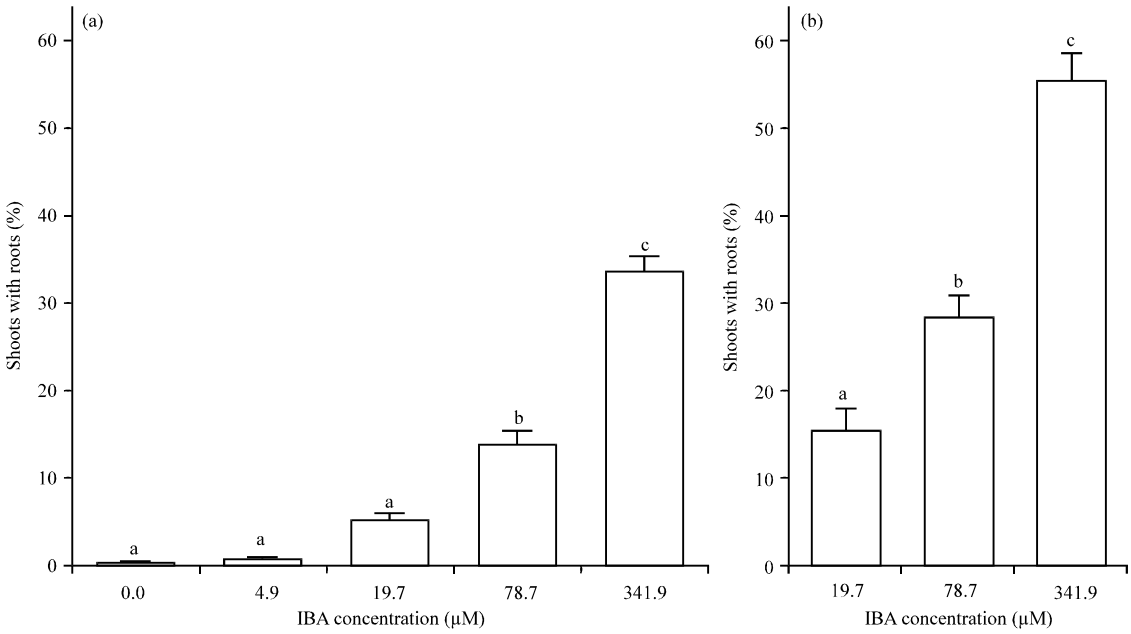

Fig. 2(a-b): Root formation *in vitro* on *Philotheca sporadica* shoots treated with different concentrations of Indole-3-Butyric Acid (IBA) after (a) Shoot proliferation and (b) Shoot elongation. Means±SE with different letters are significantly different (ANOVA and Tukey's HSD test; $p<0.05$; n = 62-71)

conditions, with 89% survival. Tissue culture is often used to rapidly propagate many plants from a limited source of explants (George, 1993; Trueman, 2006; Trueman *et al*., 2007). Tissue culture has been used to produce *Eriostemon australasius* and *E. myoporoides* plants (De Fossard and Plummer, 1981; Ault, 1994) but it had previously proven very difficult to produce *Philotheca freyciana* by tissue culture (Papworth *et al*., 2005). The current study demonstrated that, on average, over 5,000 *P. sporadica* shoots can be produced from one shoot tip in 6 months. These shoots can be converted readily into plantlets using a high IBA concentration for root induction and an IVS system (Newell *et al*., 2003, 2005; Hung and Trueman, 2012) for transferring plantlets from the laboratory to the nursery.

This study, therefore, produced *P. sporadica* plants by clonal propagation methods from 76 individuals in natural populations. A potential issue with clonal propagation methods for producing large numbers of plants for offset programs is the maintenance of sufficient genetic diversity in the offset populations. Propagation from cuttings was effective for a greater number of *P. sporadica* genotypes (63) than propagation from tissue culture (13) but tissue culture was far more effective for producing large numbers of cloned plants. Population genetics of all *P. sporadica* populations is being assessed in a companion study and the results of that study will be used to determine the most appropriate genetic composition of plants for the offset populations.

CONCLUSION

Environmental offset projects for threatened plant species are often conducted within limited time-frames that provide little opportunity for refining the propagation methods. They can be hampered by low amenability to propagation which affects both the rate of plant production and

the final number of plants available. However, identifying a successful propagation method can lead to rapid gains in production because the first-propagated plant material is often multiplied in exponential fashion to produce new plants. This study showed that *P. sporadica* could not be propagated readily from fresh seeds and it was difficult to propagate from cuttings but tissue culture was highly effective for producing thousands of plants for an environmental offset program.

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