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Paclobutrazol Trials in Commercial Micropropagation of *Grevillea* species

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Summary

Grevillea 'Bonnie Prince Charlie' is a popular landscaping plant from the Proteaceae family that is often propagated via plant tissue culture. Despite good growth and multiplication in culture, it possesses morpho-physiological characteristics that decrease its quality and survival during the latter stages of micropropagation. Excessive internode elongation and soft, thin stems exacerbate negative abiotic stress effects that occur once removed from the nurturing culture environment. Trials of the growth inhibitor paclobutrazol were undertaken to test its potential to alleviate the issues encountered during deflasking and acclimatization. Supplementation of the growth media with 2 mg/L proved a success and all problematic traits of the cultured *Grevillea* were counteracted by the application of paclobutrazol. The noted positive effects included: drastically reduced internode elongation, thickened stems capable of supporting their own weight, increased desiccation tolerance and reduced wilting, increased axillary bud growth, broader and deeper green leaves and increased consistency and density of root growth, with 97-100% of plants rooting. Outside of the laboratory environment, deflasking and acclimatization survival rates, quality of sale stock and production efficiency were all greatly improved. The only disadvantage noted was a 1-2 week increase in the holding time in the laboratory. This slight increase in passive

storage time was deemed to be a minimal trade-off in return for the multitude of advantages. Large-scale trials proved 2 mg/L paclobutrazol to be the ideal concentration to ameliorate the disadvantageous traits of *Grevillea* 'Bonnie Prince Charlie,' improving its commercial viability. Further testing with paclobutrazol has already begun on other cultured species to ascertain if there are equal improvements to be achieved.

INTRODUCTION

Plant Tissue Culture (PTC) is an asexual method of growing plants *in vitro* - a state in which plant cells, tissues and whole organs are aseptically cultured in a fully controlled environment, on chemically defined media. Plant tissue culture has a wide variety of applications, ranging from use in medicine and pharmaceuticals, scientific, horticultural, and agricultural research, species conservation to micropropagation (**Fig. 1.**).

Micropropagation utilizes plant tissue culture techniques for large scale propagation. There are several stages in micropropagation including: conditioning, multiplication, rooting (rhizogenesis), deflasking and acclimatization (ex vitro). The conditioning phase involves the harvesting and sterilization of *in vivo* plant material and culture onto media containing varying combinations of plant growth regulators to induce juvenile vegetative growth. After the plant material has been successfully initiated, the next stage is multiplication of the tissue culture plants. The last stages in the PTC cycle are the rooting phase; in which the application of auxins induces rhizogenesis and primes the plants for deflasking and acclimatization - the final stage of the cycle where the plants are removed from culture and slowly adapted to the outside environment.

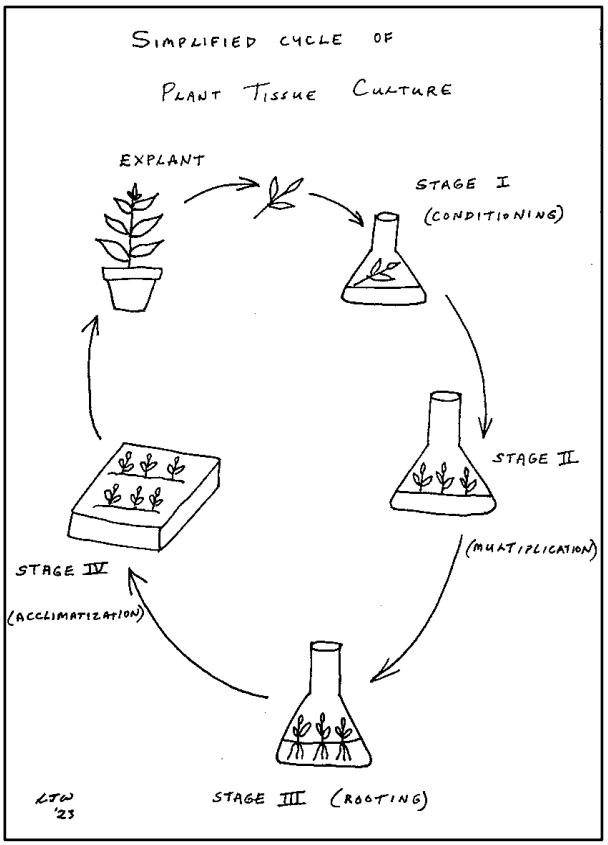

Figure 1. Diagram of a plant tissue culture cycle.

Morpho-Physiological Impediments of in Vitro Plants

The controlled growth environment triggers significant morphological and physiological changes in the cultured plants. Exogenous sources of nutrition, humidity, heat, light, growth regulators and water prompt the plants to revert to a juvenile state, with the average TC plant growing to only a few centimetres tall. Plants grown in culture become heterotrophic and are unlikely to be

actively photosynthesising, despite low levels of chlorophyll present in the leaves. The enzymes that play a role in photosynthesis (such as ATP synthase) are either inactive or absent in tissue culture plants. Carbon dioxide uptake and net exchange in cultured plants is either very slow or halted (Conner and Thomas, 1983).

Characteristics and processes in ordinary plants that help regulate water loss are vastly altered in tissue culture plants. Stomata, the pores on leaves that open and close for gaseous exchange and control loss of water vapor (transpiration), are extremely slow to close and often do not close at all. Water is readily able to escape from the vegetation of TC plants through the stomata. A less developed vascular system leads to poor water conduction between the roots and shoots of cultured plants. The leaves of plants *in vitro* do not develop epicuticular waxes in typical amounts or with the same chemical properties as plants outside of culture (Davies et al., 2018). This combination of factors put the plants at considerable risk of desiccation and death unless certain precautions are taken.

It is the combination of these characteristics which necessitate the acclimatization process. The final step of the PTC cycle serves to slowly allow the cultured plants to regain regular function and harden themselves to the outside environment. Acclimatization can be difficult even in ideal conditions, let alone when some plant species have features that make it challenging to micropropagate.

Case Focus: *Grevillea* 'Bonnie Prince Charlie'

Some species carry-over traits that make them difficult to handle in and out of culture. Woodiness, thin stems, etiolation, recumbent and prostrate habits are among many factors that can have a challenging impact on their commercial micropropagation viability. If a certain species is soft and less woody, it follows that its cultured counterpart will also be soft and less woody. *Grevillea* 'Bonnie Prince Charlie' (BPC) is among many popular *Grevillea* varieties sold commercially, but unfortunately, this variety has a fine and delicate form when initiated into culture. Stems average 0.5-1mm in diameter, in contrast to other *Grevillea* species which range from 1-2mm (**Fig. 2**).

Figure 2. A typical *Grevillea* 'Bonnie Prince Charlie'.

'Prince Charlie' cutting in tissue culture. This variety grows faster than other observed cultures at an average rate of 2cm per week. It has been suggested that plants *in vitro* excessively produce gibberellins (Roberts *et al.*, 1992), therefore, it is likely this species secretes high endogenous levels. The speedy growth and thin, fragile stems contribute to a strong wilting predisposition both *in* and *ex vitro*. The plants are also easily damaged when handling with tweezers in culture and during the deflasking process. This form and fragility, coupled with elevated water loss, means these plants are at a significant disadvantage when they leave the culture environment to commence the acclimatization process.

(slower production rates), but most critically, greater losses post deflasking and significant reduction in plant quality. Issues encountered during deflasking included damage to the plants from handling (e.g. snapping of stems), excessive wilting and deformation of plants (unable to support their own weight, stems adopted a sideways 'S' shape) and greater prevalence of fungal rot due to plants drooping over into the media (**Figs. 3 and 4**). Any plants that survive the acclimatization process will likely have an undesirable form to customers and low saleability. Techniques can be employed to help salvage the stock but are labour intensive and involve triple-handling.

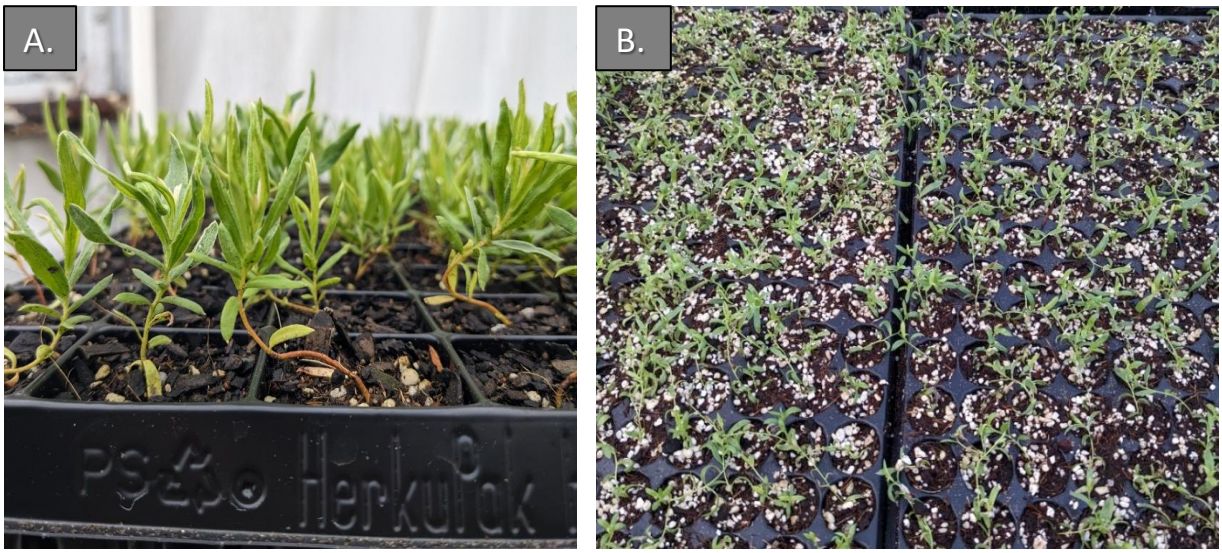

Figure 3. A) and **B).** Low quality *Grevillea* 'Bonnie Prince Charlie' cultures at various stages of hardening off - note the warped stem shapes and inability to support their weight.

Attempts to alleviate and/or resolve these problems included:

- Making size adjustments to the cuttings taken for rooting/pre-acclimatization phase: decreasing height of cuttings, harvesting stem cuttings.
- Alteration of PTC media recipes: trialling different types/combinations of plant growth regulators, different basal salts.
- Ventilation of culture flasks prior to deflasking: containers were opened slightly for one week to enable air exchange and start the acclimation process earlier.

- Minimizing plant exposure by speeding up the deflasking process as much as possible.
- Optimizing the process of deflasking: only having one open culture container at a time.
- Conducting deflasking in a greenhouse with high humidity to help minimise shock to the plants.
- Use of anti-transpirants.

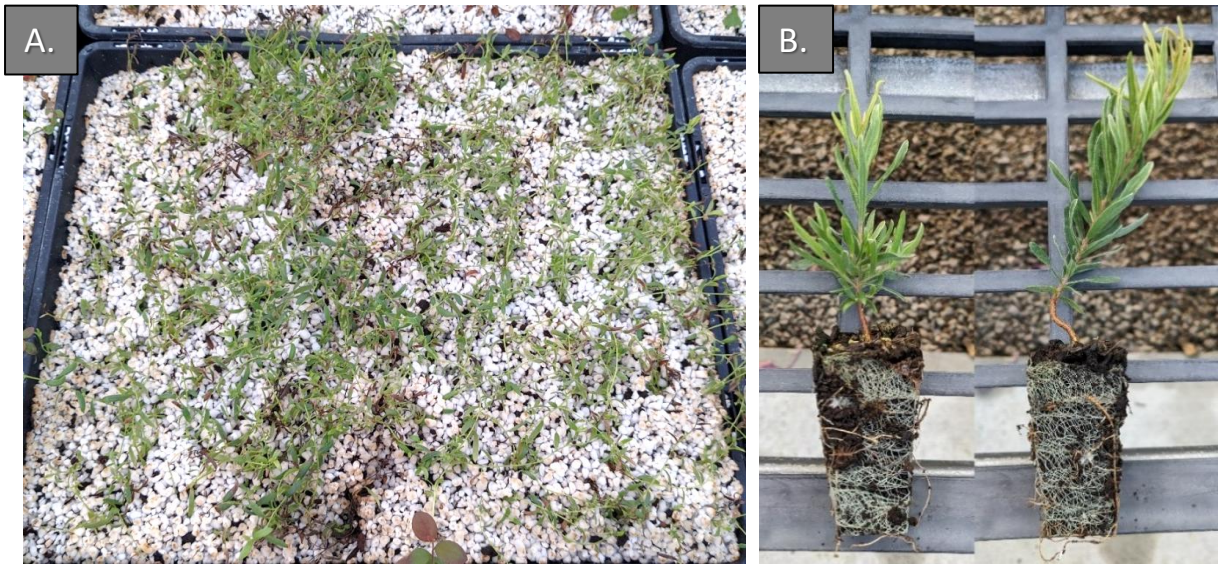

Figure 4. A) A tray of deflasked *Grevillea* BPC - sizes are inconsistent, most plants have collapsed under their own weight and there is die-off and botrytis. **B)** An example of a high quality (right) and a poor (left) quality sale stock.

Unfortunately, even in combination, none of these methods yielded significant success, however, did serve to slightly improve the survival rate of the plants *ex vitro*.

MATERIALS AND METHODS

Paclobutrazol (PBZ) is a growth inhibitor which prevents the biosynthesis of gibberellins. It can be used as a pre-treatment in the rooting phase of the PTC cycle to help overcome abiotic stresses such as water loss. It is known to reduce elongation of internodes, increase leaf quantities and thickness of stems (Abdalla et al., 2021) and act as a systemic fungicide (Desta and Amare, 2021). These qualities made PBZ an ideal trial candidate for amelioration of issues surrounding *Grevillea* 'Bonnie Prince Charlie'.

A bottle of 'Trimmit' growth regulator was sourced for the trials, containing the active constituent 250g/L Paclobutrazol. The original product was diluted to a concentration of 0.125g/L with distilled water. Extensive research culminated in a concentration of 2mg/L being chosen as the starting point for testing. As 'Trimmit' is not a product intended for use in plant tissue culture, an initial test on four *Grevillea* varieties ('Bonnie Prince Charlie', 'Sunkissed', 'Gold Rush' and 'Fire Cracker') was undertaken to screen for any potentially detrimental or fatal effects. 2mg/L PBZ was added to a rooting media pre-sterilization

and dispensed into small sterile culture containers. Of each variety, twenty plants were placed onto the trial media. No ill effects were observed on any of the trial varieties, and all had improved root growth. Rhizogenesis was faster, consistent, with more dense, thick root balls.

RESULTS AND DISCUSSION

The most notable vegetative improvement was in the *Grevillea* BPC, which exhibited deeper green, more rounded, and dense leaves, thicker, firmer stems which did not elongate further than the height at which they were cut. Axillary bud proliferation was also stimulated. There was an increase in the holding time between the rooting stage and deflasking - trial plants took an average one to two weeks longer to reach ideal form for deflasking. A small amount of trial stock was held to observe the long-term effects of PBZ. A half-life of approximately 35-45 days was discovered and varied based on plant variety and media constituents. After this period, the PBZ would wear off and internodal elongation would resume from the lower region of the plants. Some plant varieties exhibited the physiological disorder hyperhydricity (excessive hydration and malformation of tissues) after long periods of exposure to PBZ. As the trials were designed for temporary use in the rooting phase these issues were deemed to be of no consequence.

The BPC trial was deflasked and it was found that the plants were easier to handle, had better roots and rarely wilted. A second, large scale trial was conducted, in which half a batch of *Grevillea* 'Bonnie Prince Charlie' contained the 2mg/L media addition of Paclobutrazol. Flasks containing 1350 trial plants and 1350 control plants were deflasked. There were very notable differences between the trial and control plants; with the majority of trial plants maintaining a height half that of the control plants (**Figs. 5, 6,** and **7**), with thicker stems, increased quality and survival rate. The trial stock was inspected at the latter stages of acclimatization and progression throughout the nursery and was found to have no adverse effects and remained well-formed and healthy.

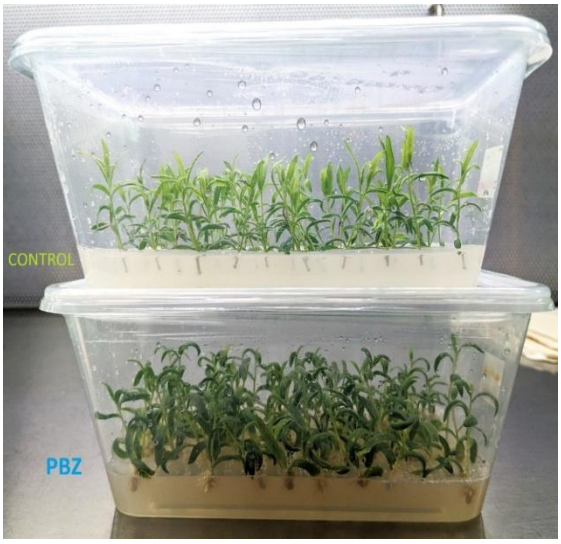

Figure 5. Trial versus control: while the control is still healthy, viable stock, it is thinner and more fragile than the PBZ trial which has deeper green, thicker, more compact vegetation and more progressed and better-quality root growth.

Effects of subsequent trials remained consistent, with significant improvement to the strength and resilience of the vegetative tissues, plus faster, more consistent, better quality root growth. As seen in the images left and below, the trial plants have a greater surface area and thickness. The control plants are more than twice the height of the PBZ trial, despite production on the same day. The control plants have warped stems due to excessive vertical growth. Smaller trials were undertaken to test different concentrations of

PBZ at 1mg/L and 1.5mg/L but were found to be too weak for the desired results. Concentrations of more than 2mg/L were not tested due to the risk of adverse effects on the plants.

Figure 6. Side by side comparison of *Grevillea* 'Bonnie Prince Charlie' trial versus control. The control plants are double the height of the trial, have poor form, and damaged vegetation due to abiotic stress.

Figure 7. A) comparison of the trial and control plants *in vitro* – there is a clear difference in the plant morphology. **B)** Heights of control and PBZ trial plants side-by-side.

Based on the success of the large-scale trial, all further batches were incorporated with

PBZ and observed closely for quality and consistency. The 2mg/L concentration remained in place as it yielded ideal results. A combined total of 9090 *Grevillea* BPC plants have been successfully produced since the start of the trials, with a substantially higher survival rate and greatly increased quality. There were also increases in productivity throughout the chain of production from the lab all the way to tubing and potting. Time spent grading the stock drastically decreased, and due to the improved resilience and quality of the plants, their progression through the nursery was fast and trouble-free. The trial stock also had a greater holding capacity, transforming *Grevillea* BPC into a low-maintenance, flexible and commercially viable product (**Figs. 8, 9,** and **10**).

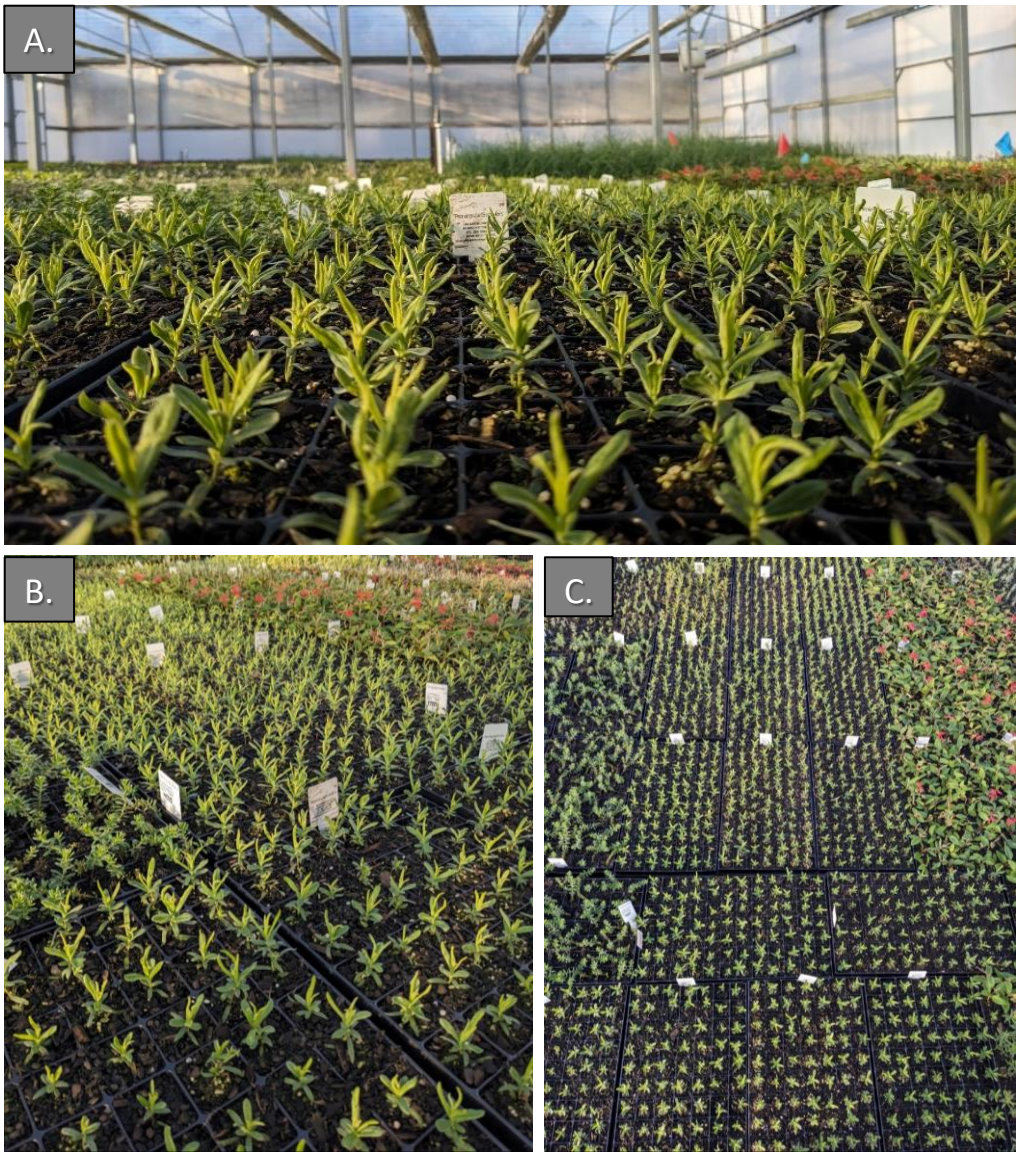

Figure 8. A) Uniform trial of *Grevillea* BPC in early stages of acclimatization. **B)** and **C)** Trial of *Grevillea* BPC in early stages of acclimatization.

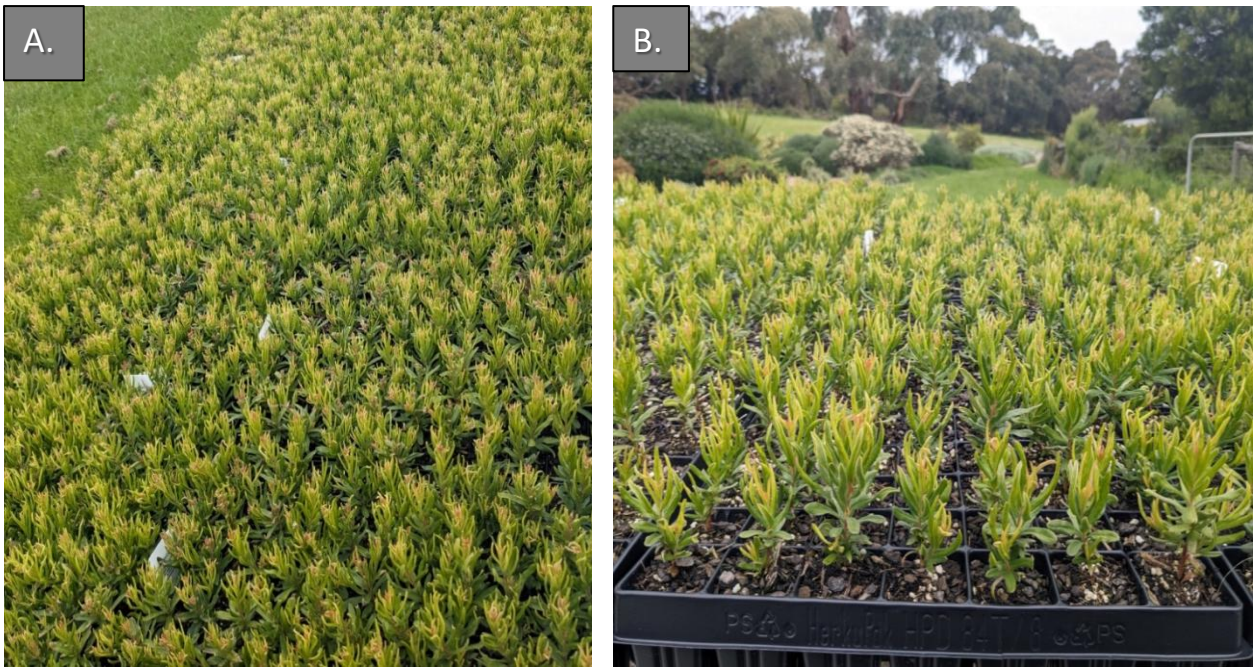

Figure 9. A) Fully hardened, high quality sale-ready stock. **B)** Ideal form of trial *Grevillea* BPC stock.

CONCLUSIONS

Grevillea 'Bonnie Prince Charlie' can be a challenging plant to propagate. While it may grow quickly and multiply well in culture, it has too many morpho-physiological characteristics that decrease its quality and survival rate when deflasked. Excessive internode elongation and soft, thin stems exacerbate the effects of abiotic stress that these TC plants are exposed to when they are removed from the culture environment. The addition of the growth regulator paclobutrazol into the PTC media to improve survival and quality of *Grevillea* BPC proved a success. All problematic traits of the TC *Grevillea* were counteracted by the PBZ.

Noted benefits included:

- thicker, straight stems, capable of supporting their own weight
- very little wilting
- increased desiccation resistance
- more axillary bud growth
- improved leaf form
- no excessive internode elongation
- more consistent rhizogenesis, ranging from 97-100% consistency
- denser root growth
- significantly higher survival rate ex vitro
- higher quality sale stock
- increased production efficiency in the laboratory and nursery

The only slight disadvantage of the PBZ addition was a 1-2 week increase in the holding time in the laboratory. Ultimately, a slight increase in passive storage time is a very minimal trade-off in return for the multitude of advantages. Further testing with paclobutrazol has already begun on other species in culture to ascertain if there are equal improvements to be achieved. Many of the trials have shown positive results thus far. More research and testing will be undertaken to investigate other potential uses for PBZ within the plant tissue culture and micropropagation fields.

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