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LARGE SCALE PROPAGATION AND IN VITRO WEANING FOR THE RESTORATION OF *VIOLA PALUSTRIS* TO SUPPORT ASSISTED COLONISATION OF A THREATENED BUTTERFLY

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Abstract

The distribution and abundance of Boloria selene *(small pearl-bordered fritillary butterfly SPBF) declined over the decades in many parts of the UK. Availability of food plants, especially marsh violet (Viola palustris), for the caterpillars of the SPBF has been identified as one of the major limiting factors for this decline. To achieve augmentation of existing colonies and develop new populations of SPBF large numbers of marsh violet propagules were required specifically to feed the larvae. The main objectives of the study were to produce thousands of good quality marsh violet propagules to restore selected habitats in the Heart of Durham, Northern England, using in vitro methods. Preliminary trials showed that in vitro multiplication of seedlings from wild collected seeds through conventional agar-based cultures was lengthy, expensive and turned out to be a non-viable route to achieve the objectives. This study explored the potential of bioreactor-based cloning and cost-effective one step rooting and weaning. Robust propagules, ready for transplantation following rapid propagation and one step rooting and weaning in vitro, were raised in a plug system for transplantation and establishment under field conditions. This was achieved by using simple and cost-effective methods to support the large-scale restoration exercise using 14,000 propagules. Application of high throughput micropropagation and low cost one step weaning systems for time-bound conservation and restoration projects are discussed in detail. This research highlights the important role of in vitro methods to support integrated biodiversity conservation of threatened native plant and butterfly.*

Keywords*: Recovery; In vitro weaning; Food plant; Photoautotrophic; Community planting*

Introduction

Serious declines in larval food plant populations, especially that of marsh violet (*Viola palustris*), is one of the reasons for the decline of small pearl-bordered fritillary (*Boloria selene*), referred to as SPBF from hereon, in the north east of England. Habitat specific SPBF has low mobility and breeds in damp and grassy areas where there are food plants such as *Viola canina* and *V. palustris* Availability of food sources is a major predictor of population abundance and this relationship is strongest among species with low mobility [1]. The SPBF was once commonly found in much of the UK but the species has undergone major population declines in both distribution and abundance in central and eastern England [2]. This study was

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conducted to address the population decline of *V. palustris* and related decline of SPBF in the Heart of Durham area. The Heart of Durham SPBF project required large numbers of the larval food plant, *V. palustris*, for assisted colonisation in farmland and wild sites across County Durham [3]. This effort championed by The Wildlife Trusts in the UK's Living Landscape initiative is a recovery plan for nature (*http://www.wildlifetrusts.org/living-landscape*) to help create a resilient and healthy environment rich in wildlife as well as improving conditions for wildlife.

In vitro methods of propagation can provide propagules for reintroduction when conventional propagation methods are inadequate [4]. Large scale micropropagation systems are now being routinely used for diverse restoration activities underpinning the success of different methods available [5-6]. *Viola* spp. have been successfully micropropagated using non-meristematic tissues as explants [7-9]. However, preliminary in vitro studies showed that agar-based multiplication, rooting and transplantation alone were not enough to produce thousands of propagules required every year for large scale transplantation from wild collected seeds for this project. Therefore, the main objectives of the current study were large scale in vitro propagation of marsh violet using wild collected seeds, development of efficient rooting and acclimatisation in vitro, and successful transplantation of propagules in selected habitats. The costs of tissue culture methods are greater than for conventional propagation, but critical for achieving tangible conservation outcomes in some species. In such cases it is important to develop methods that are efficient, both in terms of cost and time, in delivering numbers and quality of propagules. Can in vitro systems be improved to cater for the needs of policy makers and conservationists to meet the demands during a period of unprecedented biodiversity loss and decreasing financial resources?

Materials and methods

Seed sterilisation and pre-treatment

Seeds received from the Durham Wildlife Trust (DWT) were accessioned at RBG Kew as 4009, 4010, 4013, and 4014. Seeds were collected from extant populations of *V. palustris* at Waskerley Way, Pow Hill and Black Plantation – SPBF sites along the river Browney. Following a test of sterilisation timings, all seeds were surface-sterilised in a 0.5% (w/v) solution of sodium dichloroisocyanurate (NaDCC) and a drop of Tween 20 using the filter paper packet method as described elsewhere [10] for 30 minutes. Sterilised seeds were washed in a single change of sterile water, then rinsed with 50% (v/v) Plant Preservative Mixture™ (PPM™, Plant Cell Technology, USA) in sterile deionised water for 10 minutes and left to air-dry in the laminar flow bench. These seeds were given a pre-treatment with filter-sterilised solutions of gibberellic acid (GA3) at concentrations of 0, 10, 50, 100 and 300mg/L (w/v) in deionised water overnight on a rotary shaker.

In vitro culture

Murashige and Skoog (MS) medium [11] was used for all in vitro trials with pH set at 5.8 and 0.8% agar was used as gelling agent for solid culture medium. All the media were autoclaved at 121°C and 15psi for 15 minutes. Cultures were kept at 22±2°C on a 16/8 photoperiod of $20 mol \times m^{-2} \times s^{-1}$ for seed germination, $35 mol \times m^{-2} \times s^{-1}$ for vegetative multiplication, and $50 mol \times m^{-2} \times s^{-1}$ for one step rooting and weaning using cool white fluorescent lamps.

Seed germination

Treated and untreated seeds of all accessions were germinated on half strength MS medium in Cell Counter vials (25mL, Alpha Labs, UK). Activated charcoal (AC) at 0.15%

(w/v) and sucrose (2%, w/v) were also included in the germination medium. Single seeds were sown in each vial (10 mL medium) and cultured until seedlings with three leaves developed.

Shoot multiplication from seedlings

a. First round of multiplication on agar medium

Multiplication of *V. palustris* was attempted using full strength MS nutrients with 0.15% AC (w/v) and 3% (w/v) sucrose. Culturing was done in Magenta jars with B Caps (Sigma Chemicals, UK). Four seedlings with at least three fully developed leaves were transferred to the multiplication medium and incubated under conditions described as above. Data were collected on the quality and numbers of axillary bud multiplication every 4 weeks.

b. Bioreactor-mediated high throughput shoot multiplication

Cloning by temporary immersion system (TIS) called RITA® (Cirad Biotrop, France) was used for the liquid cultures. After preliminary trials for shoot multiplication with TDZ, BAP and 2iP, TDZ was selected as the cytokinin for further culture trials. Fresh multiple shoots (4 weeks old) grown on agar medium containing 0.15mg/L TDZ and either 0, 0.05 or 0.1mg/L 1-naphthalene acetic acid (NAA) were harvested for this study. Liquid medium consisted of full MS nutrients with 3% (w/v) sucrose with 0.15mg/L TDZ at pH5.8. Since liquid culturing carries greater risk of contamination than semi-solid agar culturing the liquid medium also contained 5mL/L PPM™ for the suppression of bacterial growth. For the TIS immersion cycle, explants on the platform were immersed in liquid medium (120mL) for 5 min. duration every 30 minutes using an aerating pump (Brighton Systems, UK). To achieve a fast multiplication process, explants used consisted of a clump raised on the agar initiation medium with a maximum of three fully developed shoots.

c.Second round multiplication on agar medium

Multiplied shoot clumps, developed from explanted clumps in the bioreactor, were subdivided into sub-clumps and cultured in Magenta jars containing agar-solidified medium with MS salts and vitamins supplemented with 0.05mg/L TDZ.

Cultures were grown in this 'conditioning medium' for up to 16 weeks or until shoots with leaves were fully developed that could be grown in plug trays for rooting and weaning. Remaining clumps with shoot buds were subjected to further rounds of TIS multiplication. Growth of endophytic slow-growing bacterial contamination in agar culture was controlled using 3mL/L PPM™ and sodium dichloroisocyanurate (NaDCC) as used for in vitro collecting [12]. NaDCC was added at a rate of 0.03% w/v, via stock solution, after autoclaving and once the medium had cooled but before solidification.

Single step rooting and weaning

Rooting plugs were made by mixing one-part vermiculite with one-part tissue paper pulp and 0.7 parts coir (weight/weight) in a blender by gently vortexing for 10 seconds with ½ strength Hoagland's solution [13] (Sigma Chemicals, UK). This substrate was strained using a sieve to remove excess solution so that the substrate retained sufficient moisture for plantlets' growth. The substrate was autoclaved at 121°C and 15psi for 50 minutes – the large volume of the substrate and the organic composition (coir) accounting for the increased time for sterilisation. Plastic flip-top food disposable containers measuring 190×140×70mm (w×d×h) (Polymer UK, London) were used as the rooting and weaning vessel. A longitudinal rectangular hole was made in the lid of the box and Micropore™ (Millipore, UK) tape (soaked in absolute ethanol and air-dried for sterilisation) fixed over it to improve air exchange but maintain sterility of the culture. Seventy percent ethanol was sprayed into the interior of the containers, the lids closed, and vessels left for 10 minutes. Plastic plug trays with plug well dimensions of 18×17×24mm (w×d×h) and volume of 5cm^3 (H. Smith Plastics Ltd., Essex, UK), cut to size to fit the culture vessel (35 wells per tray) were also sterilised using 70% ethanol, filled with the prepared mix under sterile conditions and plug-tray transferred to the box.

Elongated shoot cultures, cultured over four weeks in shoot elongation medium containing ½ strength MS medium (agar), with at least three leaves were transferred to individual plugs in sucrose-free ½ strength Hoagland's solution [13] and incubated under cool white fluorescent tubes at 22±2°C on a 16/8 photoperiod of 50mol×m^{-2}×s^{-1} (higher light intensity than agar cultures to account for photoautotrophic growth, i.e. the lack of sucrose in the liquid medium) for two weeks. Once the shoots had rooted and produced new leaves under photoautotrophic conditions the tape was removed and the plug trays with plantlets were transferred to shade house conditions.

Transplantation of in vitro propagules

The propagules were transplanted from the photoautrophic culture boxes in the early spring when water availability was good and average temperature was conducive to survival of propagules. Propagules from a single plug tray (maximum 35) were either transferred to 2L plastic pots (20cm diameter) with a generic compost substrate, 'community planting', or as single propagules in smaller pots. Once the propagules in the community pots had been grown for at least 5 months they were planted out into the field sites identified as part of the SPBF project. Planting areas chosen were free of standing water and dense ground cover and close to areas planted with nectar-bearing species grown for adult SPBF since the range of the adult butterfly is only around 200m [2]. Each unit of established *V. palustris* plants and compost from a 2L pot were inserted into the ground.

Data collection – vegetative propagation

Number of seeds germinated was collected after periods of 8, 12 and 16 weeks. Percentage of germination was calculated based on the total number of seeds received against total number of seeds germinated. Germination data was analysed using single factor ANOVA to ascertain whether there were differences between pre-treatments and growing media. Multiplication frequency (number of new shoots per explant) of the shoot cultures in both liquid and agar multiplication was analysed using single factor ANOVA followed by Student's t-test.

Data collection – establishment of propagules in pots before introduction to the wild

A sample of 58 pots (maximum of 35 propagules in each) was selected that had been transplanted for an average of 10 months. Number of plants established was counted, and a qualitative 'establishment rating' of growth quality and density was given of between 1 and 5, where 1 = very small plants with few leaves, low number of plants per pot and little or no obvious fresh growth and 5 = full establishment of most or all plants and profuse fresh growth filling the pot. Data on number of plants established per pot regarding number of bioreactor cycles were gathered, grouped according to accession, and analysed by single-factor ANOVA followed by t-test.

A 5m×5m quadrat was used at the sites to select plantings to record. For each planting, 4 measurements were taken: module spread per pot (i.e. distance between furthest leaf tips of module x the equivalent perpendicular measure); % ground cover (i.e. in plan-view, percentage cover of marsh violet leaves in spread area versus either other plants or bare ground); number of flowers per community pot; number of fruits per community pot. Plantings over two years were assessed to analyse the progress of establishment in the field. Additionally, data were gathered on percentage ground cover for a small, naturally occurring population of marsh violet near one of the plantation sites (Black Plantation). This allowed comparison of introduced and natural plants. Module-spread and percentage ground cover were analysed by single factor ANOVA followed by t-test.

Results and Discussions

All stages of in vitro processes, from seed germination to development of transplantable propagules, and transplantation to field are shown in detail in Figure 1.

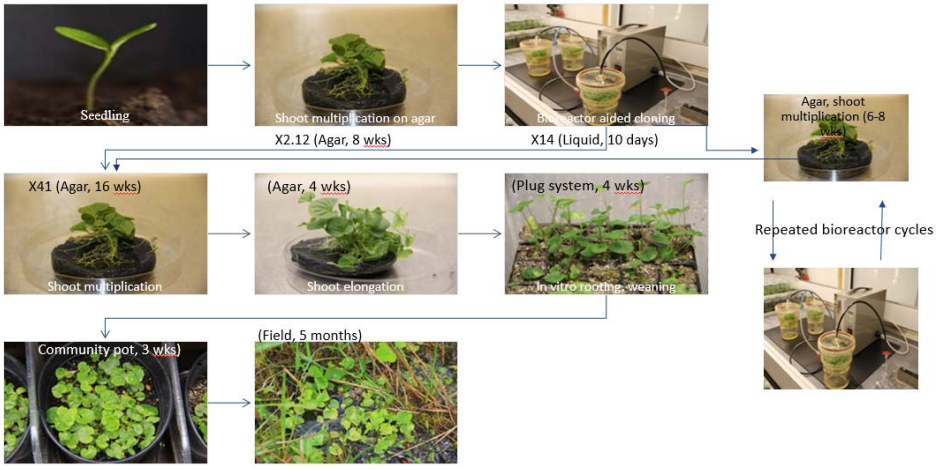

Fig.1. Flow chart explaining stages from seed germination to transplantation to the field

Seed germination

Due to the lack of available seeds collected from the wild only 520 seeds of two accessions were available for the study. The two accessions, 4009 and 4010, were sown following overnight soaking in water with different concentrations of GA3. Sample sizes were insufficient to complete statistical analysis for this test, therefore, GA3 pre-soaking concentration was evaluated based on data available from Millennium Seed Bank (MSB). The accessions 4013 and 4014 that were soaked in 300mg/L GA3 on basal medium germinated and were used for further trials. A combined figure of 10.2% seed germination after 6 weeks was achieved from 1333 seeds.

V. palustris seeds are dispersed by explosive dehiscence which makes seed collecting from the wild very difficult and added to this is the low quality of seeds developed on extant plants in the study area. Seed germination tests at the MSB [14] used gibberellic acid to improve germination in water agar which yielded 95% germination. The seeds collected from selected locations in the Heart of Durham behaved differently from the previously reported results, probably due to low quality or low genetic diversity of the seeds. However, several plants raised from in vitro seed germination produced fruits in culture through self-pollination of which 18 germinated in vitro (22.2%). Previous reports suggest that *V. palustris* seeds have very high viability [15], however, the germination rate in this study was low presumably due to the poor quality and variability of seeds collected from wild populations.

Vegetative multiplication

Initial tests for agar multiplication showed poor growth of seedlings in media containing various types and concentrations of cytokinin except on thidiazuron (TDZ) containing medium. Total average multiplication rate was 2.12 after 8 weeks. For further multiplication on agar, basal medium was supplemented with 0.15mg/L TDZ.

For faster multiplication, the temporary immersion system, TIS, RITA® was used. Shoots were cultured for 10 days in the TIS producing clumps of tissue containing small shoots and buds. When transferred these clumps grew, multiplied further and elongated on fresh basal

agar medium supplemented with 0.05mg/L TDZ. Endophytic bacterial contaminants are a serious bottleneck in liquid cultures and eradicating them completely to successfully clone plant material is a challenge. Endophytic contamination of *Ilex dumosa* cultures was eradicated using an antibiotic in a bioreactor system [16]. Our initial sterilisation process was to minimise bleaching damage to the viable embryos as only limited seed stocks were available. This led to seed batches germinating and producing healthy 'clean' seedlings, but some shoot cultures were contaminated by bacteria after a couple of cycles while developing stock shoot cultures in preparation for bioreactor cycles. However, by using 5ml/L PPM™ we have successfully controlled the contamination problem. PPM™ has been successfully used in culturing many taxa that are difficult to sterilise and when small amount of material is available [17-19]. Antibiotic use is common practice for crops and rare plant micropropagation [19-20] to eliminate bacterial contamination. However, antibiotics were not used in this study because the propagules raised were to be used as food plants by the larvae. Additionally, use of antibiotics in conservation related tissue culture activities cannot be considered as an appropriate practice. Propagules raised in antibiotic/s-containing media should be avoided in native plant conservation, especially those that are used in reintroduction and restoration activities. Successful eradication of endophytic bacteria was observed in black kangaroo paw (*Macropidia fuliginosa*) using PPM™ [21]. Plant regeneration varied between taxa, type of explant and range of PPM™ used on three crops [22]. In chrysanthemum percentage of explants forming shoots and the number of shoots formed were significantly reduced [23]. There was no quality deterioration as a result of PPM™ usage in our study and the cultures were contaminant-free in liquid cultures.

Data were gathered to investigate the differences in multiplication rate between liquid culture and agar culture. The new shoots were counted 4, 8, 12 and 16 weeks after the first bioreactor cycle. The use of the RITA® system markedly improved multiplication rate of the plants (Fig. 2).

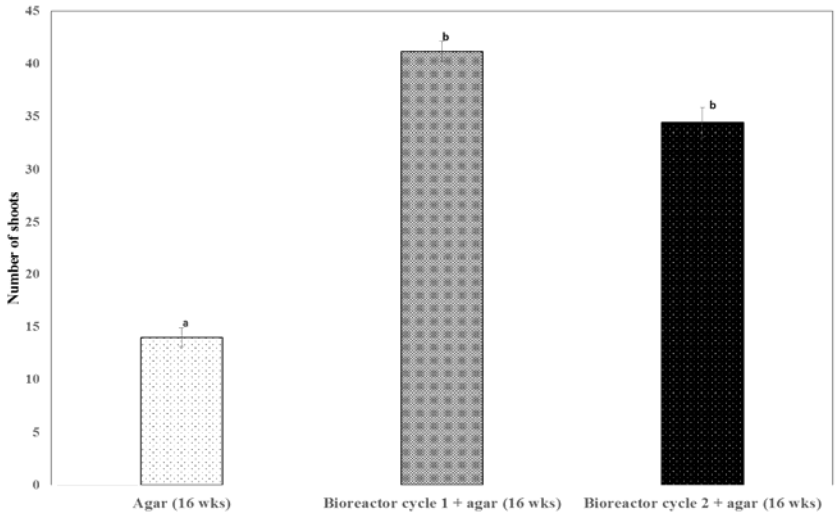

Fig. 2. Multiplication rate for *Viola palustris* cultured in semi-solid agar and liquid immersion system. 1 – Agar multiplication alone; 2 – multiplication using RITA®; 3 – multiplication using a second RITA® cycle. Bars represent means of at least 28 explants +/- standard error after 16 weeks. Bars with the same letter are not significantly different (P>0.05).

After 16 weeks of growth, one cycle, on agar based medium explants produced an average of 14±0.89 shoots. However, after a single bioreactor cycle of 10 days plus 16 weeks in

agar an average of 41.2±0.95 shoots were produced. After a second bioreactor cycle, over 10 days, 34.4±1.41 shoots were produced after 16 weeks (Fig. 2). This means significant increase was noticed when cultures were grown in bioreactor before multiplication on agar medium. Additionally, there was no significant difference in shoot number between multiple bioreactor cycles.

Use of propagules from non-meristematic tissue through either callus mediated or direct organogenesis has intrinsic risks in producing plants of altered genotypes. Tissue culture-regenerated propagules are susceptible to somaclonal variations as described in *Clivia miniata* [23] whereas direct regeneration from shoot tips yielded less variation in propagules compared to propagules raised from leaf and petals through indirect organogenesis. To avoid potential pitfalls with seed set and recruitment our objective was to propagate the species using meristematic regions of the seedlings, both apical and axillary meristems. Previous studies on propagation of *Viola* spp. were based on regenerating propagules from non-meristematic tissues [7-9]. Only shoot tips and axillary meristems were used for clonal multiplication in this study to minimise risks of potential somaclonal variation. Plants were screened for flowering, seed-set and germination and we noticed no changes in morphology and phenology in the clonal propagules. Flow cytometry analysis of clonal in vitro propagules showed no difference in ploidy compared to the original seed-derived progenies. Several approaches are available for in vitro propagation and their use for different applications. Reintroduction and assisted colonisation require diverse, high quality propagules [24] but the in vitro methods required for these applications must be selected prudently to preserve the genetic integrity of the material in the wild germplasm of the selected species [25].

Another of key finding of our study was the quality of shoot cultures produced with no apparent presence of hyperhydration. Previously, hyperhydration has been reported during multiplication in liquid media-based large-scale multiplication [26-27]. Juvenile explants of *Rhodiola rosea* multiplied well in a bioreactor system but hyperhydrated shoot cultures had to be grown on agar medium to produce healthy shoots [26]. Similar results were observed in Chinese water chestnut where a bioreactor system was used for cloning [27].

Single step rooting and weaning

The shoots harvested from the bioreactor were grown for four weeks in the shoot elongation medium (Fig. 1) for the single step rooting and weaning trials. Conventional agar-based media containing low concentrations of minerals and low or no sugar, resulted in poor rooting (Fig. 3A and B) and none of the transplanted plants survived under glasshouse conditions.

Due to the lack of appropriate facilities for handling of in vitro raised propagules at Low Barns (DWT, Durham) where the propagules were planted out, 'in vitro pre-weaned' propagules were prepared at RBG Kew. A sterile plug system was used [28], with sucrose-free ½ strength Hoagland's liquid medium, as a photoautotrophic system to improve rooting and weaning of propagules so that minimal handling would be necessary during transplanting. Roots produced using the plug system were numerous and robust following culture of the elongated shoots grown on ½ strength MS medium (Fig. 1, Fig. 3C and D).

Photoautotrophic micropropagation using sugar-free medium is widely used based on the success of producing relatively high photosynthetic abilities of plantlets in vitro [29]. Our study revealed that uniform high-quality propagules can be raised through this system which needs minimum or no transition period between removal from the photoautotrophic system and survival ex vitro.

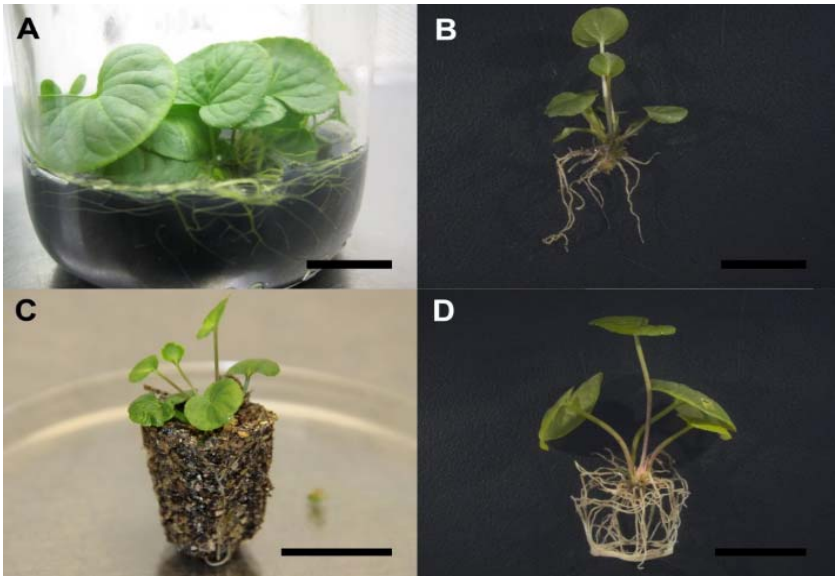

Fig. 3. Improved rooting of in vitro *Viola palustris* propagules using photoautotrophic plug substrate and sucrose-free liquid nutrient medium. A – agar culture; B – roots of agar-grown propagule; C – photoautotrophic plug propagule; D – photoautotrophic propagule with substrate removed to show robust root growth. Scale bars 1cm

Shoot proliferation of *Gypsophila paniculata* in a bioreactor and its rooting in a microponic culture system were efficient methods which helped to produce high quality plantlets and reduced production costs [30]. The rate of multiplication, rooting and acclimatization has a major effect on the cost for producing propagules. Substantial improvements that will increase the efficiency of these steps can help lower costs [31].

When resources for conservation are spread for ever increasing lists of threatened plants, available funds for recovery, propagation and restoration are limited. Lengthy labour-intensive methods are costly and were cited as bottlenecks necks in delivering success in using in vitro propagules for autumn buttercup conservation [4]. There are many reports that highlight the potential application of bioreactor technology to aid conservation [32-35]. However, to our knowledge there is no published report yet on using a bioreactor system to mass propagate native plants for large scale restoration of butterflies.

Transplantation and translocation trials

Although different methods of transplantation of in vitro plug propagules into ex vitro conditions were trialled on site the best outcome was obtained by transplanting all plug propagules from a whole tray into a single two litre pot containing compost, 'community planting' as described before. After at least 5 months' pots were assessed for survival of plug propagules and establishment ratings attributed (1-5 where 1 is very poor establishment with few good leaves and 5 is fully established with luxuriant, dense leaf growth) (Fig.4).

Number of plants established was counted, and a qualitative 'establishment rating' of growth quality and density was given of between 1 and 5, where 1 means very small plants with few leaves, low number of plants per pot and little or no obvious fresh growth and 5 means full establishment of most or all plants and profuse fresh growth filling the pot.

Data were analysed for number of plants established and establishment rating for a representative sample of in vitro propagules in boxes transplanted to two litre pots. No significant difference was observed ($P > 0.05$) between seed accessions for either parameter.

Establishment was also tested by number of bioreactor cycles to assess whether deterioration in establishment occurred for propagules that had undergone extra bioreactor cycles. No significant difference was observed in either parameter for propagules that had undergone multiple bioreactor rounds.

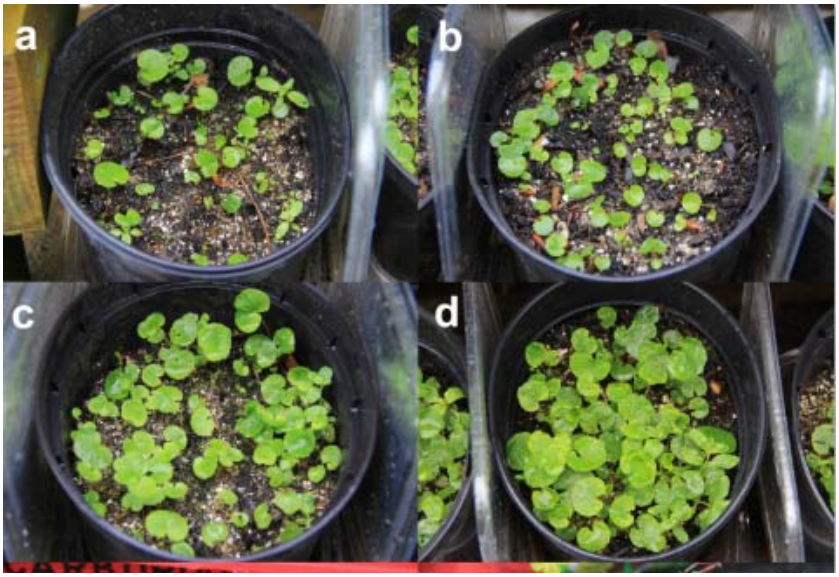

Fig. 4. Establishment rating measure for *Viola palustris* five months after potting up from photoautotrophic plug trays.

Average establishment rating for all measured pots was 3.8 with average number of plug propagules established per tray was 23. Average number of propagules produced per tray in vitro had been 32, therefore overall establishment from in vitro to establishment in pots under field conditions was 72%. This is a very satisfactory return given the absence of specialised horticultural facilities on site and inexperience of staff and volunteers handling the plug propagules. Our results show that 'community planting' improved survival rate compared to propagules transplanted individually. It was noted that extant populations, *V. palustris* grows in tight clumps of multiple shoots and this method of transplantation replicates natural growth.

Once plants in pots had acceptable growth they were planted out at the field sites. The spread and percentage cover of each planted community pot varied across the different sites when analysed after four months and 16 months (Fig. 5). These figures were based on averages in the quadrat over a minimum of five community pots sampled at Byerley House and 16 at Stuartsfield sites.

Within planting years there were no significant differences ($P > 0.05$) in module spread or percentage cover for each community pot planted. Percentage ground cover was higher in the 2015 plantings ($P < 0.05$) than the 2014 plantings, possibly due to reduced competition from surrounding vegetation. Both 2014 plantings occurred in tree plantation areas and may have had a lower light level and nutrient availability. Additionally, Stuartsfield site was found to have a drier soil while Black Plantation had a waterlogged soil with little drainage. Both Middles Farm and Byerley House sites appeared more suitable for the plants, with wet but well-drained soil, inclined ground and higher light levels with less tree shading and this observation supports the previous findings- The coppice rate fell by 90% in actively managed woodlands in England between 1905 and 2000 [36]. As a result, many woodland gap-dependent species declined

during this period, including the *Viola*-feeding fritillaries [37]. In our study the sites also seemed more suitable given their higher incidences of fruiting. Percentage ground cover was significantly higher ($P < 0.05$) for all planting sites versus the naturally occurring population at Black Plantation site. Higher percentage ground cover suggests that introduced plants produced a good number of healthy new leaves that were allowing the plants to establish well, particularly at Byerley House and Middles Farm sites.

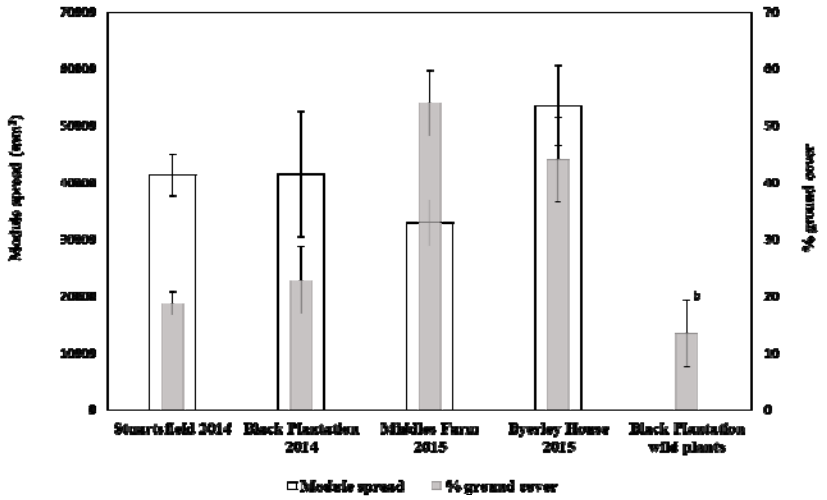

Fig. 5. Growth and establishment of ex vitro *Viola palustris* propagules in field sites after 16 months (2014 plantings) and 4 months (2015 plantings). Bars represent the average from a minimum of five colonies +/- standard error. b = significantly ($P<0.05$) lower ground cover than all other sites.

The 'community planting' model used in this study with 14,000 plants in pots and their subsequent transfer to selected assisted colonisation sites during spring helped establishment of plants. Linking different assisted colonisation sites, mainly farmland, with wild sites in this study has the potential to develop connectivity corridors for marsh violets to recruit naturally and thereby expand the occurrence of SPBF. Recovery programmes that aim to establish new wild populations of species face fresh challenges in the age of climate change [38].

As the geographic ranges of some rare taxa have become increasingly restricted conservation intervention and/or restoration requires highly effective systems [39]. Attempts to reintroduce species back to their original wild habitats or introduce them to different areas (translocation/assisted colonisation) requires an assessment of the suitability of potential sites [40-41] as success is linked to climatic suitability [42]. We think the systems we used, propagules developed, and the strategies adopted to establish sustainable colonies of food plants for SPBF were suitable and robust for the large-scale nature of the project.

Conclusion

Complex layers of lengthy, expensive propagation processes, loss of propagules during acclimatisation, and very low establishment under field/wild conditions are driving away policy makers and conservation practitioners from using in vitro methods. Our research showed that high throughput cost effective in vitro methods underpin the success in large scale propagation of genetically diverse germplasm. Simultaneously, propagules raised via in vitro systems should

be resilient and easy to handle by untrained people for successful establishment. Ultimately, the results of this study will lead to the successful establishment of populations of both marsh violet and the small pearl-bordered fritillary butterfly as preliminary results are very promising. This shows smart, time-bound and low-cost methods could successfully support large scale projects in a changing climate where biodiversity loss is a serious issue.

Acknowledgments

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