IN VITRO PROPAGATION OF *WAHLENBERGIA STRICTA* (R.BR.) SWEET SUBSP. *STRICTA*, AN ADVENTIVE PLANT IN NEW ZEALAND, CAN YIELD PLANTS CAPABLE OF FLOWERING AND SETTING VIABLE SEEDS

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ABSTRACT

Carson, J.A. & Leung, D.W.M. (1997). *In vitro* propagation of *Wahlenbergia stricta* (R.Br.) Sweet subsp. *stricta* an adventive plant in New Zealand, can yield plants capable of flowering and setting viable seeds. *New Zealand Natural Sciences* 23: 87-91.

Micropropagated *Wahlenbergia stricta* (R.Br.) Sweet subsp. *stricta*, an adventive New Zealand plant, could form flowers when maintained under *in vitro* conditions or after exflasking (i.e. growing in soil under glasshouse conditions). Comparison between flowers of *in vitro* plants and those kept in the glasshouse revealed similar developmental patterns although *in vitro* flowers were one third smaller in size. Apparently viable pollen were produced by the clonal *W. stricta* plants whether they were kept *in vitro* or exflasked. However, seed set was only observed in exflasked *W. stricta* clonal plants. The seeds thus obtained could be germinated and the seedlings grown to maturity (flowering).

KEYWORDS: Blue bell - *Wahlenbergia stricta* - *Wahlenbergia trichogyna* - rare plants - *in vitro* culture (flowering).

INTRODUCTION

Wahlenbergia stricta (R.Br.) Sweet sub-species *stricta* (formerly *W. trichogyna* Stearn) is an untidy straggling herb with prominent purplish blue bell-shaped flowers 2.0 - 3.5 cm in diameter, with a deep bell of 7 x 5 mm becoming pale yellow at its base. Fruits are globe-like capsules, also with short hairs and 5 prominent vertical ribs 5 - 7 mm x 5 mm, containing many minute seeds (Allan 1961, Wilson & Given 1989).

In a recent study (Carson & Leung 1994b), *W. stricta* was successfully micropropagated from one of two plants found in New Zealand in 1991. Little other work has been done on this plant except for one study carried out on temperature and light sensitivity in relation to other grassland species of Eastern Australia (Willis & Groves 1991). Also, some relatives within the genus have been studied for allozyme variation and their evolutionary relationships in Juan Fernandez Islands (Crawford *et al.* 1990), and on Campanulaceae, flower physiology (review by Shetler 1979) and potential crossing experiments (Nyman 1991).

A novel observation of our previous study (Carson & Leung 1994b) was that the clonal plants of *W. stricta* could form flowers under *in vitro* conditions. In this study, we compare the development of flowers by clonal plants maintained under *in vitro* conditions and those grown on in the glasshouse. Furthermore, the question of particular interest is whether the clonal plants derived from the original *W. stricta* plant in 1991 are capable of setting viable seeds.

MATERIALS AND METHODS

The medium used for plant growth contained 3% sucrose (w/v) dissolved in half strength Murashige & Skoog (1962) basal

medium (MS). The pH of the media was adjusted to 5.7 then 0.8% (w/v) agar added and dissolved by microwaving to boiling. The solution was then dispensed into clear plastic tissue culture containers in 20 ml aliquots (Intermed Scientific Ltd, Takapuna, NZ) before solidification. Sterilisation by autoclaving for 14 min. at 137kPa completed media preparation. Subculture involved excising developing shoot tips and flower or terminal buds from previous subcultures and inserting the stems 3 to 4 mm into the media for an upright stance. Cultures grew at 22 °C in a growth room with lighting of 65 to 85 μmole m^{-2}sec^{-1} provided by Philips TLD32W/84HF fluorescent tubes (Philips, Holland) and incandescent bulbs for 23 hours a day. Continuous light from Sylvania Growlux F30W/T8/GRO tubes (Sylvania, Germany) overcame the hour break in main lighting while it reset. For the study of *in vitro* flowers, it was necessary to harvest flowers at different stages of development for measurement.

The *in vitro* plantlets can also be exflasked (i.e. removed from tissue culture containers) and established successfully in soil under glasshouse conditions (Carson & Leung 1994b).The developmental stages of established glasshouse plants flowering in planta bags were: small green bud, large green bud, contoured purple bud, petals separating, petals folded, flower open, stigma split and flower wilted. Measurements included size of calyx and petals, and appearance of stigmas and anthers. A comparative study of the flowers formed by *in vitro* plantlets and those by exflasked plantlets was then made.

Seed from *ex vitro* *W. stricta* material growing in the glasshouse was pooled together over a period of two months. This material was then soaked in 100 μg l^{-1} GA$_3$ for 36 hours. The seed was then immersed in household bleach containing 5% (v/v) NaOCl as determined by the MCD assay (Hager *et al.* 1966) for 10 minutes. Seed was planted at five per container on 3% (w/v) sucrose supplemented half strength MS under standard culture conditions.

Pollen from both *in vitro* and *ex vitro* flowers was processed for scanning electron

microscopy (Neil Andrews, University of Canterbury, pers. comm.) using an electron scanner (Cambridge Stereoscan 250 MKII, Cambridge Instruments Ltd, Cambridge, England).

Pollen viability was determined using the staining method of Alexander (1969). Pollen germination was measured over several fields of view under a stereo microscope. Germinated and ungerminated pollen was observed after 0, 12, 24 and 36 hours into the experiment. Germination was defined as the presence of a pollen tube longer than the diameter of the pollen. *W. stricta* pollen from newly opened flowers was used. Each treatment involved one replicate and each experiment was repeated once.

RESULTS

Both *in vitro* flowers and those from exflasked plants (referred to as *ex vitro* flowers here) exhibit the same developmental sequence of characteristic morphological changes from the appearance of immature floral buds to the eventual senescence of petals (Table 1). It is however, notable that the developmental sequence of *in vitro* flowers takes just over 2 weeks, a much shorter time scale than *ex vitro* flower development ($P<0.0001$). Another notable difference is that the average size of fully open *in vitro* flowers is only half that of *ex vitro* flowers ($P<0.0001$, Table 2). Petal length of fully open *ex vitro* flowers contributes the most, continuing to grow after opening which results in a correspondingly large difference in total flower length (average size). Earlier stages of flower development described in Table 1 were also measured but with insignificant results (data not shown).

In vitro flowers appear to function normally, producing similar pollen to *ex vitro* flowers. When the mature floral bud opens, pollen is present along the hairy sides of the style right up to the stigma tip. As the flower develops further, the top of the cylindrical style peels back revealing the stigma which splits into three lobes. The self pollen (from the same flower) did not appear to reach the stigma surface.

Scanning Electron Microscopic (SEM)

Table 1. Comparison of developmental stages between *in vitro* and *ex vitro* flowers of *W. stricta*. The mean number (± standard error) of days a flower takes to reach subsequent developmental stages from day one where the bud has just developed.

Flower Development	*In vitro*	*Ex vitro*	ANOVA
Green bud with top of corolla dome below highest point of calyx	1	1	
Green bud with top of corolla dome above highest point of calyx	3.9 ± 0.49	8.4 ± 1.06	$F_{(1,60)} = 223.04$ $P < 0.0000$
Purple contours as bud reaches full size; petals of corolla turn purple and grooved	7.6 ± 0.79	22.8 ± 0.99	$F_{(1,64)} = 1530.36$ $P < 0.0000$
Folded petals around enclosed stamen, stigma and style assembly; petals lengthening	10.9 ± 0.69	25.7 ± 1.43	$F_{(1,72)} = 731.47$ $P < 0.0001$
Open split stigma in 3 lobes slowly wilts to coil back on itself	14.5 ± 0.58	36.2 ± 1.30	$F_{(1,16)} = 1004.86$ $P < 0.0001$
Senescence when petals wilt	17.7 ± 0.76	39.0 ± 1.28	$F_{(1,45)} = 5041.00$ $P < 0.0001$
Seed/open polyp, 3 holes appear in top from which seed spill	-	54.5 ± 2.44	

observations reveal no apparent differences in the morphology of pollen of *in vitro* or *ex vitro* flowers. The pollen is spherical with a diameter of about 38 μm. Its surface is covered with thin needle-like structures. Two to three apertures of 8 μm diameter were spaced equidistantly around its surface. A plug-like operculum fills the inside of the aperture, protruding slightly above the pollen surface.

Using Alexander's staining method (Alexander 1969), the pollen from *in vitro* and *ex vitro* flowers is apparently 100% viable. However, limited success is achieved with attempts to germinate both *ex vitro* and *in vitro* pollen on water-agar supplemented with various additives including sucrose, boric acid, $CaCl_2 \cdot 2H_2O$, yeast extract and 2,4 D. To date the best result is a pollen germination rate of 17% from just opened floral buds after 24 h at 22°C in water-agar media supplemented with 10% (w/v) sucrose and boric acid at either 10 or 100 mg l^{-1}. No seed set is seen with *in vitro* flowers following petal senescence, although in our SEM study a germinated pollen with its pollen tube was found in the vicinity of the stigmatic surface of an *in vitro* flower, suggesting the seed set by *in vitro* flowers might be possible. The *ex vitro* flowers exhibit a low frequency of seed set (13.2% of the 105 flowers studied) in a glasshouse. Following petal senescence, the ovaries of these flowers either immediately turn pale, or swell and develop a red and green stripped vertical pattern on the ovary wall. Those pale ovaries which fail to swell collapse, dying back to a branch in the stem. In contrast, the swollen ovaries then either turn pale or redden slightly as the ovary dries, forming a seed capsule. Most flower heads wilt and the petals, stigma and style assembly drop off. Gentle shaking or the effects of wind blowing the 30-40 cm long flower stalks releases seeds from 3 holes in the top of the capsule. The seeds are elliptical, shiny brown or black with a smooth surface. Seed size is no more than 1 mm in diameter, weighing 21 ± 7.4 μg. Average seed production per flower is 33 seeds, but varied between 2 and 168 seeds.

Without pre-sowing soaking in GA_3(100 μg ml^{-1}) the *ex vitro* flower seeds fail to germinate. About 60% of the seeds germinate under axenic *in vitro* conditions following a 48 h soaking in 100 μg ml^{-1} GA_3. After one month, the 6-8 cm tall seedlings are transferred to media with or without 3%

Table 2. Comparison of mean flower size (± standard error) between *in vitro* and *ex vitro* plants of *W. stricta*. Flowers were sampled when fully opened with stigma split in its three lobes. Lengthways measurements were made from the ovary base to the calyx tip, sepal length, petal length and total length from ovary base to petal tip.

Flower Development	*In vitro* size (mm)	*Ex vitro* size (mm)	ANOVA
Ovary Length	4.4 ± 0.51	4.1 ± 0.35	$F_{(1,21)}= 1.77 \ P = 0.1972$
Sepal Length	5.6 ± 0.52	7.6 ± 0.63	$F_{(1,21)} = 57.17 \ P < 0.0001$
Petal Length	8.3 ± 0.46	27.1 ± 1.90	$F_{(1,21)} = 733.9 \ P < 0.0001$
Total length	18.1 ± 0.64	38.9 ± 1.96	$F_{(1,21)} = 832.68 \ P < 0.0001$

sucrose. Three weeks following the transfer, 75% of the plants in the sucrose-containing medium have flower buds. Another month later, these flower buds mature into flowers and numerous immature flower buds are also present. In contrast, the plants on sucrose-free media have on average one floral bud for every two plants, and remain bright green and large leafed after the same period.

DISCUSSION

Few previous reports of *in vitro* flowering have attempted to compare various aspects of flower formation of *in vitro* and *ex vitro* plants. Here, we showed that the flower structure and pollen morphology of *in vitro* flowers of *W. stricta* are very similar to the flowers of the glasshouse-grown plants.

W. stricta was able to continually initiate flowers following subculture of the micropropagated clonal plantlets. However, this *in vitro* flowering ability was initially absent in the *W. stricta* seedlings derived from seeds of the clonal plants. That is, when the seedlings were grown under identical *in vitro* conditions, they did not form flowers in the same time frame (3 weeks) of a typical subculture of micropropagated clonal plantlets. Flowering in the plants developed from the seedlings under *in vitro* conditions took place much later. Thus, it appears that during successive subcultures of the micropropagated plantlets, the flowering condition is passed on and maintained. This has also been suggested in a study of *in vitro* flowering in *Leptinella nana* (Carson & Leung 1994a).

A difference between *in vitro* and *ex vitro*

W. stricta flowers was size. Fully open *in vitro* flowers were found to be half the size of their *ex vitro* counterparts. This is typical of *in vitro* flower buds, controlled by adding hormones to aid development although not used in this study (Tran Thanh Van 1973, Scorza 1982, Dickens & van Staden 1985, Lee *et al.* 1991). Limited numbers of *W. stricta* pollen from *in vitro* and *ex vitro* flowers germinated on a medium containing low concentrations of boric acid normally found in flower tissue (Vasil 1964) and high sucrose (also associated with flower induction). Inadequacies of the media used may be one of the reasons for the lack of success with pollen germination.

The production of germinable seeds by *ex vitro* *W. stricta* plants indicated that *W. stricta* was capable of inbreeding since the *ex vitro* plants were all cloned from one seedling (Carson & Leung 1994b). The flowers were not hand pollinated, and conditions of the glasshouse prevented some normal vectors pollinating the flowers. This may have accounted for the low numbers of flowers pollinated, and the subsequent reduction of seed produced.

The method of seed dispersal in *W. stricta* plants seems to rely on a combination of wind and long flower stems. The seed capsule dries, developing three holes at the top of the ovary as it matures. The long stems sway in the wind, catapulting seeds from the open capsule. The smooth shape and size of seed is also compatible with this hypothesis.

No seed was produced from *in vitro* *W. stricta*, although attempts had been made to hand pollinate the *in vitro* flowers. One possible explanation is the failure of the ovary

to desiccate. In *W. stricta* the seed capsule dries as it develops *ex vitro*. The enclosed tissue culture container prohibits the drying process and may hinder seed development as the dampness could be seen when the ovary matured. Perhaps the use of semi-permeable membrane in the culture container will help reduce the humidity.

In the previous studies (Burritt & Leung 1991, Carson & Leung 1994a,b) we proposed that *in vitro* propagation of rare or endangered New Zealand plants has the potential to aid restoration of these species in the wild. The present investigation on *W. stricta*, although not valued as rare or indigenous anymore, provides further evidence to support this contention as the *in vitro* plantlets not only established in soil but also developed to maturity (flowering) and went on to produce germinable seeds.

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