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# *In vitro* bulblet production of *Brunsvigia undulata* from twin-scales

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**Abstract**

Many South African medicinal plants are over-collected for use in traditional medicines. This necessitates developing methods for increasing production. Micropropagation can be used as an alternative to conventional propagation methods. Twin-scales, cut from large parent bulbs, were cultured on MS medium (Murashige and Skoog, 1962) supplemented with 25 plant growth regulator combinations. Bulblets formed on twin-scales in 24 of the treatments. All explants formed bulblets on plant growth regulator-free medium. The effect of plant growth regulators, activated charcoal, explant orientation, explant origin and photoperiod on bulblet production was investigated. Bulblet formation was greatest when twin-scales were excised from the middle of the parent bulb, placed adaxial side down on plant growth regulator-free medium and kept in a 16 h photoperiod.



*Keywords:* Amaryllidaceae; Micropropagation; Twin-scales

## 1. Introduction

*Brunsvigia undulata* F.M. Leight. (Amaryllidaceae) grows in the grasslands of KwaZulu-Natal, South Africa. The bulb is used to treat a number of ailments by the Zulu, Xhosa and Southern Sotho peoples. The unsustainable harvesting of these plants for the traditional medicinal trade threatens wild populations of *B. undulata*. *In vitro* propagation via twin-scale explants may allow for rapid and cost effective production of these plants, decreasing the pressure on wild populations. The plant is also favoured for the horticultural trade. The large and attractive blooms in several shades of red and pink are desirable additions to gardens and rockeries (Du Plessis and Duncan, 1989). Species such as *Brunsvigia undulata* have a fan arrangement of leaves which adds to its aesthetic appeal. It was proposed that a number of *Brunsvigia* species will make good container and/or feature plants (Pooley, 1998).

*Brunsvigia* plants are sensitive and quite difficult to grow (Pienaar, 1994). The recalcitrant seeds must be sown soon after harvest as they are only viable for a short time. Seedlings of the dwarf species can take up to four years to flower (Du Plessis and Duncan, 1989; Pienaar, 1994) and they have long generation times (Fennell and Van Staden, 2004). Larger species will mature for six or seven seasons before they flower (Du Plessis and Duncan, 1989). They are a genus for the patient gardener.

For the Amaryllidaceae, twin-scale explants which comprise of two adjacent scales connected by a piece of basal plate tissue have been successfully used for reproduction (Fennell and Van Staden, 2004). Many bulbous species successfully produce adventitious shoots from tissue at the base of bulb scales (Hussey, 1986; Robb, 1957), and from the junction of the scales on the basal plate (Fennell and Van Staden, 2004; Han et al., 2005). In the Amaryllidaceae, it is necessary to include the basal plate as part of the explant as no bulblets are formed if not present (Fennell and Van Staden, 2004). When the basal plate is cut, apical dominance is overcome and the out-growth of pre-existing axillary meristems are stimulated (Fennell et al., 2001).

There are many factors which affect organogenesis *in vitro*. Many of these factors e.g. plant growth regulators, photoperiod, explant polarity and origin from within the parent bulb, were investigated in this study utilizing twin-scale explants. Perhaps the most important of these, plant growth regulators, generate different types of organogenesis depending on the type and ratios

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used (Yeoman, 1986). Activated charcoal, when added to tissue culture medium, promotes the growth of the tissue explant in culture, due to its adsorptive properties (Pan and Van Staden, 1998; Peck and Cumming, 1986; Weatherhead et al., 1978) and its role in darkening the culture medium (Wang and Huang, 1976). Explants may react differently depending on which of their surfaces is in contact with the medium (Fennell and Van Staden, 2004) and morphological differences between inner, middle and outer bulb scales suggest that twin-scales from these areas may behave differently in culture. Bulblet formation from twin-scales has been achieved under both light (Jacobs et al., 1992) and dark conditions (Colque et al., 2002) suggesting that the optimum photoperiod is species specific.

The aim of this research was to develop and optimise a tissue culture protocol for the formation of bulblets from twin-scale explants of *Brunsvigia undulata*.

## 2. Materials and methods

*Brunsvigia undulata* F.M. Leight. plants were collected from along the road to Mount Gilboa forestry estate (29° 16.764′ S, 30° 17.627′ E) in early February 2007. Whole plants were dug up after flowering and seed dispersal, but before leaf senescence. The bulbs collected were approximately 10 cm in diameter. All leaves were removed from the plants and the bulbs were washed in tap water to remove sand or soil. The roots and a thin layer of the basal plate were removed and the top two thirds of the bulbs was cut away. The brown outermost scales were peeled away from the bulbs to expose the white inner scales.

Peeled and trimmed bulbs were treated with 1% (w/v) Benlate® (Du Pont, Delaware, USA. Active ingredient: Benamyl(benzimidazole)) for 15 min after which they were washed with sterile distilled water. Bulbs were decontaminated for 15 min in 0.2% (w/v) mercuric chloride with a few drops of Tween 20® (UNILAB, Krugersdorp, RSA) and then rinsed with sterile distilled water to remove the sterilant. Bulbs were cut in half and soaked in 0.1% (w/v) mercuric chloride with a few drops of Tween 20® for 10 min after which they were washed with three changes of sterile distilled water. Decontamination of bulbs was also attempted using 2.6% (v/v) sodium hypochlorite, however, this was unsuccessful and so mercuric chloride was used for decontamination.

Decontaminated bulbs were dissected longitudinally into six segments. Twin-scales joined by 5 mm of the basal plate were excised from these segments. For all but the explant orientation experiments, twin-scales were placed adaxial side down onto sterilized Murashige and Skoog (1962) medium (MS).

Unless otherwise stated, explants were placed on 10 ml of MS (Murashige and Skoog, 1962) medium with 0.1 g/l *myo*-inositol, 8 g/l agar (Agar Bacteriological-Agar No. 1, Oxoid Ltd., England) and 3% (w/v) sucrose, in 40 ml culture tubes which were sealed with metal caps and after inoculation a 1 cm wide strip of Parafilm®. The culture medium was sterilized by autoclaving for 20 min at 121 °C and 103.4 kPa. Cultures were kept at 25±1 °C under Osram® 75 W cool white fluorescent tubes in a 16 h photoperiod with an intensity of 74.4 μmol $m^{-2}$ $s^{-1}$. Unless otherwise stated, 25 twin-scales were used per treatment.

To determine the effect of plant growth regulators on bulblet formation, five concentrations of 6-benzylaminopurine (BA) (0, 2.22, 4.44, 8.87, 44.38 μM) were combined systematically with five concentrations of α-naphthalene acetic acid (NAA) (0, 2.69, 5.37, 10.74, 53.70 μM) and included in the medium.

The effect of explant orientation was established by placing twin-scales abaxial side down, adaxial side down and upright on the sterilized plant growth regulator-free MS.

To determine the effect of explant origin, 25 twin-scales were excised from three separate areas of two parent bulbs. Between 12 and 15 twin-scales were excised from each position in each bulb. The inner (Position 1), middle (Position 2) and the outer (Position 3) areas are distributed within the bulb as shown in Fig. 1.

Twin-scales were placed on medium including the same plant growth regulator concentrations as in the plant growth regulator experiment. Explants were placed under continuous (24 h) light conditions to determine the effect of the photoperiod on bulblet formation.

Twin-scales were also placed on medium including 5 g/l activated charcoal. Activated charcoal darkened the medium. The medium was supplemented with combinations of 0, 4.44 and 8.87 μM BA and 0, 5.37 and 10.74 μM NAA. These plant growth regulator concentrations were selected to cover the lower range of plant growth regulators tested in the plant growth regulator concentration experiment. The highest plant growth regulation concentrations from the above mentioned experiment were not included in the activated charcoal experiment as the earlier experiment showed that very high concentrations were not as effective as lower concentrations.

Bulblet induction was recorded as the percentage of twin-scales that formed bulblets (irrespective of number) in each treatment. The number of bulblets per explant was recorded as the number of bulblets which were produced by each bulblet producing twin-scale in each treatment.

Once bulblets had a diameter of 3 mm or more and had at least one root they were removed from culture. All medium was washed from the bulblets and their roots with distilled water. Bulblets were subsequently rinsed in 1% Benlate for 5 min. Rinsed bulblets were planted in a 1:1 mixture of perlite and vermiculite. This planting mixture was watered with 1% Benlate®. Bulblets in trays were placed in a mist house at the botanical gardens at the University of KwaZulu-Natal, Pietermaritzburg for 14 days. The mist house was kept at a

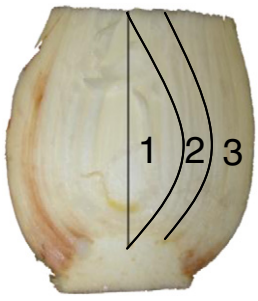


Fig. 1. Diagram indicating the positions from where twin-scales were excised from the bulbs of *B. undulata*.

temperature of 22±2 °C and at a relative humidity of 85%. Plants were mist-treated for 5 s every 5 min over a full 24 h cycle in a 16 h photoperiod at an intensity of 46 μmol $m^{-2}$ $s^{-1}$. Once bulblets had acclimatized they were moved to a green house and placed alongside a wet wall for a further 14 days, at a temperature of 24±1 °C.

Where results were recorded as percentages, the data were arcsine transformed prior to statistical evaluation (Scott et al., 1984). An analysis of variance (ANOVA) was carried out on all data. Data were analysed using a Duncan's test at the 5% level in SPSS (Statistical Package for the Social Sciences), version 10.0.

## 3. Results and discussion

Bulblets formed from the basal plate tissue. In most cases they developed between the two scales. Callus also formed on some of the explants in each of the treatments.

Bulblet induction occurred in all treatments except with 0 μM BA: 53.70 μM NAA (Fig. 2). In *Crinum moorei* Hook.F. twin-scales formed bulblets with all plant growth regulator combinations (Fennell, 2002). The highest bulblet induction was seen on the plant growth regulator-free control medium, indicating that plant growth regulators were not essential for bulblet regeneration from twin-scales. This agrees with results for many other Amaryllidaceae species, namely *Pancratium maritium* L. (Dragassaki et al., 2003), *Narcissus tazetta* L. (Steinitz and Yahel, 1982), *Amaryllis belladonna* L. (De Bruyn et al., 1992), *Cyrtanthus elatus* (Jacq.) Traub (syn. *Vallota purpurea* (Aiton) Herb.) (Kukułczanka and Kromer, 1988), *Crinum macowanii* Baker. (Slabbert et al., 1993), *Nerine bowdenii* Watson. (Mochtak, 1989). This is also true for bulbous species from other families e.g., *Lilium auratum* L. and *Lilium speciosum* L. (Takayama and Misawa, 1979). The high rate of bulblet induction may indicate that the explant contains high enough endogenous concentrations of the plant growth regulators to induce bulblet formation (Maesato et al., 1994). BA and NAA, alone or in combination, decreased the number of twin-scales which formed bulblets compared to the control.

In the absence of BA an increase in NAA concentration had a negative effect on the percentage of explants which formed bulblets (Fig. 2). Shoot and bulblet formation on twin-scales of *C. moorei* (Fennell, 2002), and *Pancratium maritimum* (Dragassaki et al., 2003) was inhibited by the presence of auxin alone. At low concentrations of BA an increase in NAA concentration caused a decrease in bulblet induction (Fig. 2).

Low concentrations of BA and NAA (2.22:2.69 μM) showed bulblet induction higher than all other treatments but lower than the control (Fig. 2). A 1:1 ratio of BA:NAA at the above concentrations was also the optimum treatment for bulblet induction in *Eucomis zambesiaca* Baker. (Ramogola and Fennell, 2007). An acceptable bulblet induction rate was also achieved by a high concentration (44.38 μM) of BA alone.

Bulblet induction was relatively high, ranging from 25% to 50%, when there was a greater BA to NAA ratio in the growth medium. An increased cytokinin to auxin ratio was conducive to bulblet induction from twin-scales of *Narcissus asturiensis* (Jordan) Pugsley. (Santos et al., 2002). BA increased shoot formation from twin-scales in *Lilium longiflorum* L. (Han et al., 2004). With high cytokinin concentrations and NAA present, up to 20 bulblets may be produced by *Narcissus* L. twin-scales (Hussey, 1978). Substantial bulblet induction (37.5%) was observed when the maximum concentrations of BA and NAA were used in combination. The greatest number of shoots as well as the greatest percentage of explants producing shoots in *Cyrtanthus* Aiton. species was achieved with the highest concentrations of both NAA and BA (Angulo et al., 2003).

A high NAA to BA ratio inhibited bulblet production by twin-scales. An increase of NAA greater than 0.05 μM reduced regeneration by twin-scales of *Lilium* L. (Maesato et al., 1994).

The greatest number of bulblets per explant was obtained by twin-scales on medium with a high NAA to BA ratio and on a medium with a greater BA to NAA ratio (Fig. 3). Both high NAA to BA ratios and elevated BA to NAA ratios successfully

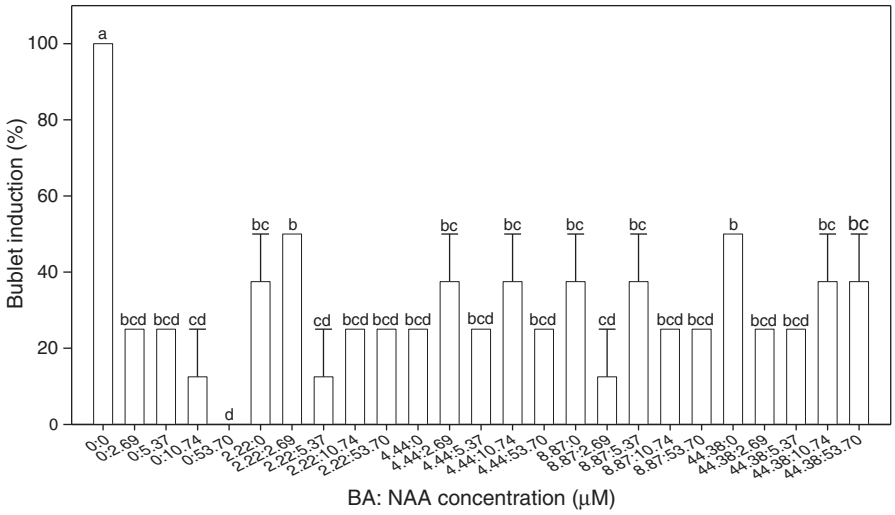


Fig. 2. Percentage of twin-scale explants of *B. undulata* which formed bulblets on medium containing different concentrations of BA and NAA. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

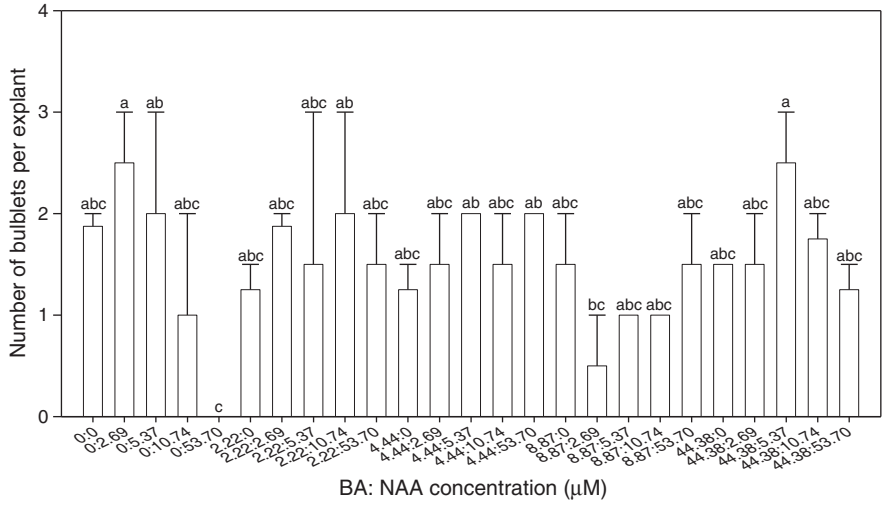


Fig. 3. Number of bulblets produced per twin-scale of *B. undulata* on the medium supplemented with different concentrations of BA and NAA. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

formed large numbers of bulblets in other Amaryllidaceae species. A high NAA to BA ratio increased bulblet production in *Nerine* Herb. (Pierik and Ippel, 1977). High ratios of auxin to cytokinin (Chow et al., 1992) and high cytokinin to auxin ratios (Hussey, 1978) increased bulblet formation in *Narcissus*.

Figure 4 clearly shows that explant orientation had an effect on the regeneration potential of twin-scales from *Brunsvigia undulata in vitro*. De Bruyn et al. (1992) found that explant orientation in *Amaryllis belladonna*, also from the Amaryllidaceae, had no effect on the regeneration potential of the explant.

In the parent bulb a twin-scale is positioned within the bulb so that it is upright. It would follow that twin-scales would be best placed in culture in this position. This was not the case when the effect of twin-scale orientation on bulblet formation was investigated. Twin-scales with their adaxial side in contact with the medium were the only ones to form bulblets. It is imperative, however, that the basal plate is sufficiently in contact with the medium. Shoots and bulblets form from the basal plate so it holds that the basal plate must have sufficient access to the nutrients in the medium (De Bruyn et al., 1992). The nutrients and plant growth regulators in the medium must be available to the competent tissues (George, 1993).

Twin-scales placed in culture with the basal plate embedded in the medium did not produce bulblets. Shoots produced by the twin-scales are produced at the basal plate. When twin-scales are placed upright in the medium the basal plate is embedded in the medium, restricting oxygen availability (Fennell, 2002; Pierik and Rubing, 1973). This prevents the basal plate from producing shoots or bulblets.

Twin-scales of *Brunsvigia undulata* are best placed in culture with their adaxial side in contact with the medium. This allows for bulblets to form both between the scales and on the abaxial surface of the explant.

The differences in the number of bulblets formed by twin-scales from different positions in the bulb were not statistically significant (Fig. 5). Twin-scales excised from the inner-most

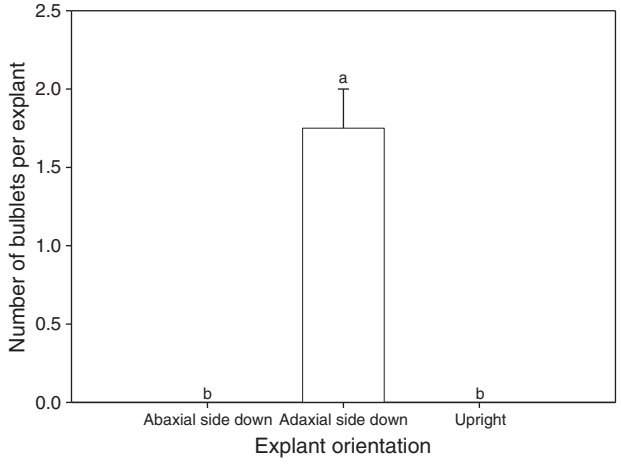


Fig. 4. The effect of explant orientation on the number of bulblets produced by twin-scales of *B. undulata*. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

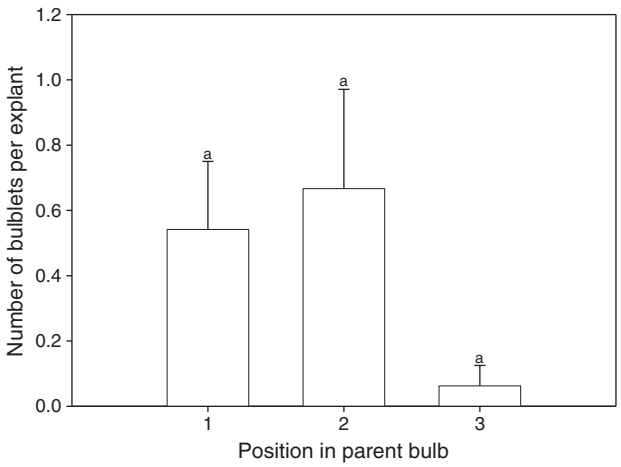


Fig. 5. The effect of explant position within the parent bulb of *B. undulata* on the number of bulblets which it produces. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

part (Position 1) of the parent bulb were very thin and fragile. The thinnest twin-scales tended to dry out in culture, reducing their ability to form bulblets. Colque et al. (2002) reported that the inner twin-scales were most productive in *Eucrosia stricklandii* (Baker) Meerow.

Twin-scales from the middle part (Position 2) of the parent bulb were the most successful in generating bulblets. Twin-scales from this position formed 0.7 bulblets per explant. Twin-scales from this position in the parent bulb also formed the most bulblets in *C. moorei* (Fennell, 2002). The twin-scales from this position are thick and fleshy. It is likely that they have greater food reserves than thinner scales. Myodo and Kubo (1952; cited by Takayama and Misawa, 1980) found differences in the levels of soluble nitrogen and sugars between inner and outer scales of *Lilium*. Scales in the middle position of *Narcissus* contained high levels of soluble and insoluble sugars (Hanks, 1986). This may explain why bulblet production was highest by twin-scales from Position 2.

A single bulblet was produced by one of the twin-scales taken from the outer part (Position 3) of the parent bulb. This explant may have been the twin-scale which was on the interface between Position 2 and Position 3. Colque et al. (2002) noted that the outer scales are those which are in direct contact with the sterilant during decontamination. In this experiment a very strong sterilant (mercuric chloride) had to be used to decontaminate parent bulbs. Mercuric chloride could damage the outermost bulb scales and their basal plate tissue preventing them from forming shoots *in vitro*.

The results of this experiment suggest that the outer part of the parent bulb has little or no regenerative potential *in vitro*. Twin-scales from Positions 1 and 2 are good sources of twin-scale explants for bulblet formation *in vitro*.

In general, twin-scales cultured in a 16 h photoperiod had a higher bulblet induction rate than those cultured in continuous light (Fig. 6), with the exception of three treatments in continuous light. Twin-scales on 53.70 μM NAA and without BA, on 2.22 μM BA and 5.37 μM NAA, and those on 8.87 μM BA and 2.69 μM NAA in continuous light had a higher bulblet induction rate than twin-scales on the same plant growth regulators but in a 16 h photoperiod. Bulblet formation is stimulated by a period of darkness in many bulbous species (Van Aartrijk and Van Der Linde, 1986). Higher rates of regeneration were obtained by exposure to darkness in *Fritillaria imperialis* L. (Witomsaka and Lukaszewska, 1997). A 16 h photoperiod was the optimum for bulblet formation by *Fritillaria thunbergii* Miq. (Paek and Murthy, 2002).

Twin-scales under 16 h light and 8 h dark produced more bulblets per explant than twin-scales, on the same medium, under continuous light (Fig. 7). A greater number of bulblets were formed on twin-scales of *Cyrtanthus* (Morán et al., 2003) and *Lilium* species (Varshney et al., 2000) in a 16 h photoperiod. Bulblet formation was greater in *Nerine* when twin-scales were exposed to a period of darkness (Pierik and Ippel, 1977). This was seen in all but two treatments. The differences, however, between the number of bulblets formed in these two treatments in different photoperiods is very small.

The present results suggest that twin-scales require exposure to a dark period for increased bulblet induction and production. As bulbs are soil-bound they are exposed to little, if any, light in their natural habitat. Dark conditions simulate the underground conditions in which bulbs normally grow (Ulrich et al., 1999).

The presence of activated charcoal increased the number of bulblets produced per explant compared with explants on medium without it in two treatments (0 μM BA: 10.74 μM NAA and 4.44 μM BA: 0 μM NAA) (Fig. 8). However, these differences were not significant. The inclusion of activated charcoal in the growth medium stimulated bulblet formation in *C. moorei* (Fennell, 2002), *Cyrtanthus* (Morán et al., 2003), *Eucrosia* Ker Gawl. (Ziv and Lilien-Kipnis, 2000), *E. stricklandii* (Colque et al., 2002), *Lilium* (Takayama and Misawa, 1980), *L. longiflorum* (Han et al., 2004), *Narcissus* (Langens-Gerrits and Nashimoto, 1997; Ziv and Lilien-Kipnis, 2000) and *Nerine* (Han et al., 2005).

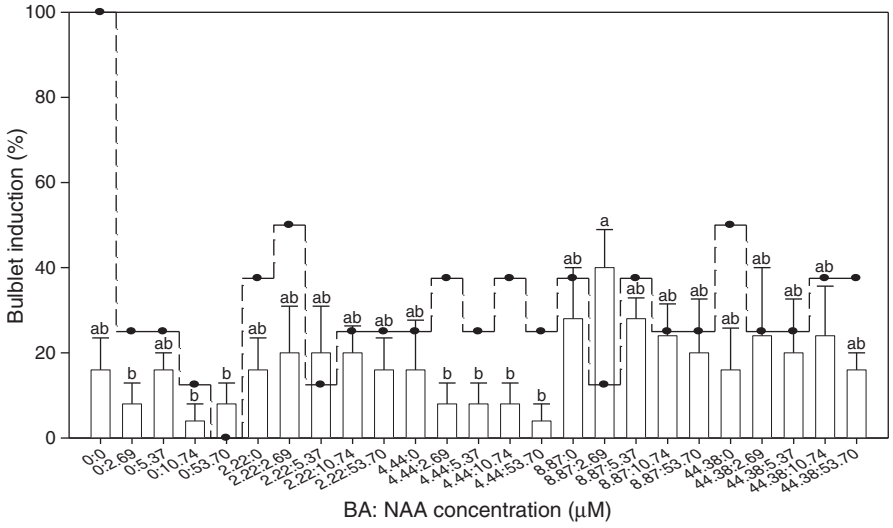


Fig. 6. The percentage of twin-scale explants of *B. undulata* which formed bulblets on varying concentrations of BA and NAA when placed under continuous light conditions. The broken line indicates the percentage of twin-scales which formed bulblets in a 16 h photoperiod. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

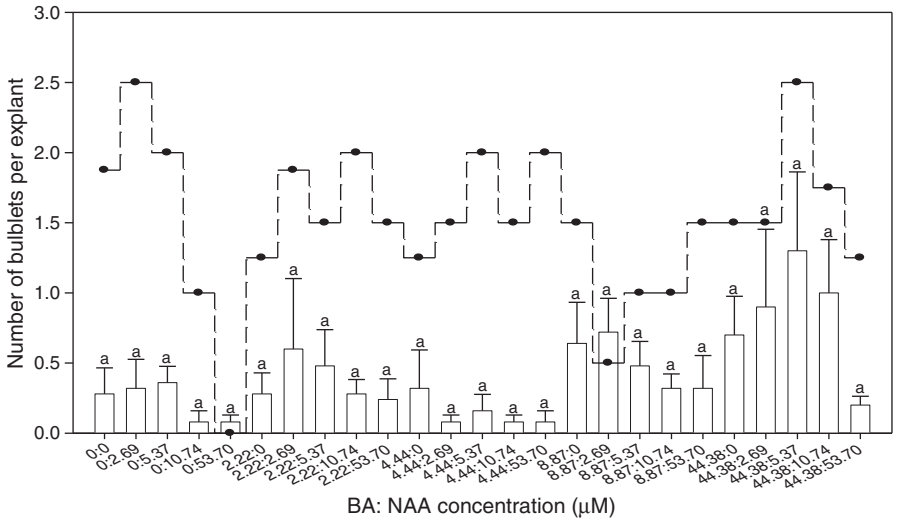


Fig. 7. The number of bulblets produced by twin-scales of *B. undulata* on varying concentrations of BA and NAA when placed under continuous light conditions. The broken line indicates the number of bulblets produced by twin-scales grown under 16 h photoperiod. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

Enhanced bulblet formation in these treatments may be due to a darkening of the growth medium, thereby creating conditions which more closely resemble soil conditions (Pan and Van Staden, 1998; Weatherhead et al., 1978).

Activated charcoal has been shown to adsorb growth substances, such as NAA and BA from tissue culture medium. This renders these plant growth regulators less effective (Pan and Van Staden, 1998; Weatherhead et al., 1978). The effects of NAA and BA will thus not be exhibited by the explants in culture. The activated charcoal may also adsorb the plant growth regulators present in the explant. Thus no plant growth regulators will be available to the explant. This may explain the lack of bulblet formation by twin-scales on medium supplemented with activated charcoal.

Figure 9 shows the percentage of bulblets in different size classes which survived after 42 days *ex vitro*. Small bulblets (3–6 mm) had the greatest percentage survival, while medium-sized bulblets (6–9 mm) had a slightly lower survival rate. The differences between these two size classes are not statistically significant. None of the larger bulblets survived *ex vitro*.

Figure 10 shows the increase in diameter by bulblets in different size classes after 42 days *ex vitro*. The greatest increase in diameter was seen by bulblets in the smallest size class. Poor increase in diameter was observed by medium-sized bulblets. Large bulblets did not survive. The differences between the three size classes is not statistically significant.

Twin-scales were most successful as explants when they were excised from the middle of the parent bulb, placed adaxial side down on plant growth regulator-free medium and kept in a 16 h

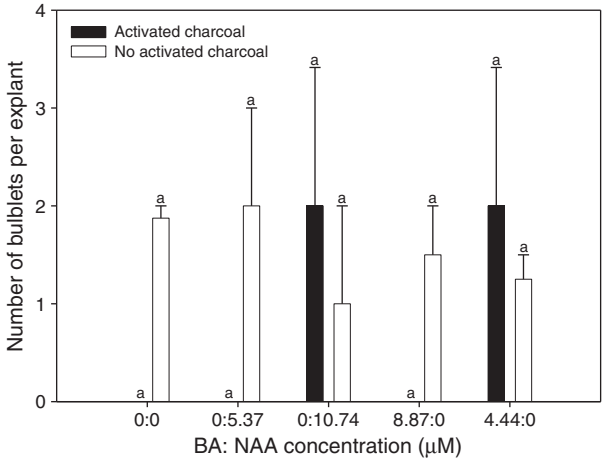


Fig. 8. Number of bulblets produced by twin-scales of *B. undulata* cultured on MS medium supplemented with and without activated charcoal and supplemented with different concentrations of BA and NAA. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

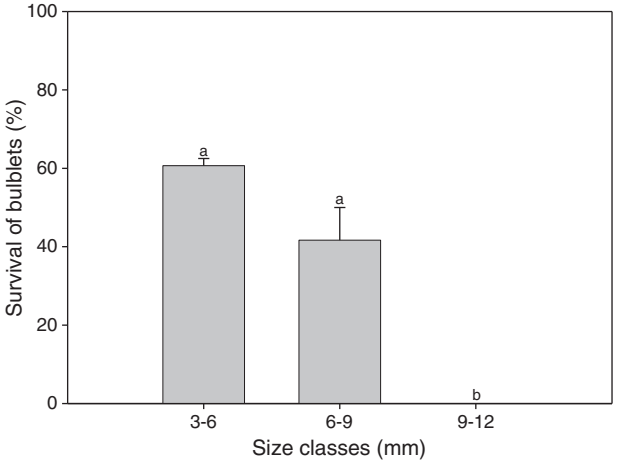


Fig. 9. Percentage survival of bulblets of *B. undulata* in different size classes after 42 days *ex vitro*. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

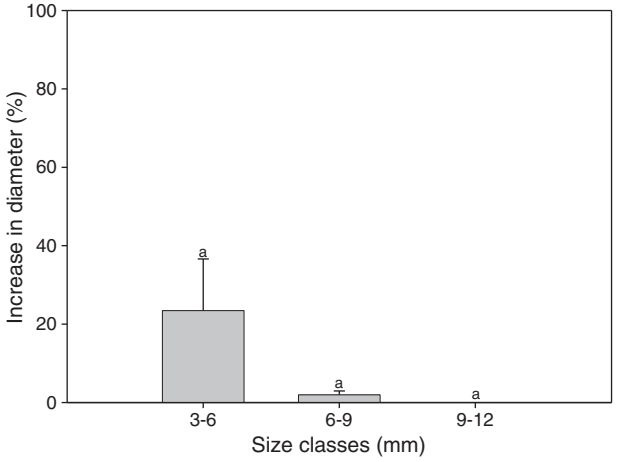


Fig. 10. Increase in diameter of bulblets of *B. undulata* in different size classes after 42 days *ex vitro*. The bars indicate standard errors. Different letters indicate significant differences between treatments at the 5% level (ANOVA).

photoperiod. This protocol allows for rapid bulblet production from twin-scales, as approximately 250 twin-scale explants may be excised from a large parent bulb, a minimum of 250 bulblets may be obtained from one bulb. Bulblets in culture underwent normal growth and development while in culture. The bulblets formed were quick to acclimatize to greenhouse conditions and grew well once planted out.

## Acknowledgements

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