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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

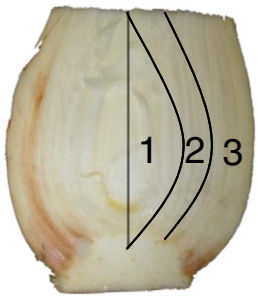

Fig. 1. Diagram indicating the positions from where twin-scales were excised from the bulbs of *B. undulata*.