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A simple and effective method for the micropropagation and *in vitro* induction of polyploidy and the effect on floral characteristics of the South African iris, *Crocosmia aurea*

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ABSTRACT

South Africa is home to approximately 10% of the world's flora, many of which are endemic to the country. A large number of South African genera have been improved for horticultural use and many of these are economically important as cut flowers or ornamentals on international markets. The genus *Crocosmia*, an attractive member of the family Iridaceae, has potential both as an ornamental plant and for cut flower production, although market potential of the species may be increased by improving the size of the flowers and inflorescence. Polyploidy has been used as a tool in the improvement of ornamental plants and has led to the development of several improved ornamental species. This study established a micropropagation protocol for *Crocosmia aurea*, using seed as the source material. Tetraploidy was induced by treating seeds with colchicine. These seeds were subsequently germinated and multiplied *in vitro* using the established protocol. The resulting tetraploid plantlets were successfully hardened-off and used to study the effect of the induced tetraploidy on the plant characteristics. The tetraploid (4n) plants were found to have longer, wider leaves as well as longer inflorescence stems and fewer, but larger, flowers than their diploid (2n) counterparts. These polyploid selections have potential in the ornamental/floriculture trade.

1. Introduction

The family Iridaceae comprises 65 genera and over 2000 species, of which 38 genera and more than half the species occur in South Africa. The plants are cormous or rhizomatous and have sword-like leaves. In general, the flowers are attractive and many species are important garden ornamentals across the globe. Although numerous species of the family have been improved by plant breeders throughout the world (Niederwieser et al., 2002; Ascough et al., 2009), there are many more species with potential for further horticultural development. *Crocosmia aurea* is no exception. This iris has bright orange-red flowers which appear from January to June on a branched inflorescence, following which a fleshy seed capsule containing purple-black seeds develops. The plant is wide-spread in the eastern parts of South Africa, where it occurs predominantly in moist areas from the coast to 2000 m above sea level (Pooley, 1993). Owing to its inherent beauty, *C. aurea* was selected for investigation of its potential as an ornamental plant and for cut flower production.

Conventional breeding has resulted in substantial improvement of genera within the family Iridaceae and many of these, such as *Gladiolus*, *Iris* and *Freesia* are important cut flowers on both local and international markets. The development of polyploid (chromosome doubling) induction protocols offers enormous potential for further improvement in the family. Naturally-occurring polyploidy is a phenomenon that has provided an important pathway for evolution and speciation in plants. Although the first polyploid was discovered over a century ago, the genetic and evolutionary implications of polyploidy are still being elucidated (Yang et al., 2011). The relative ease with which artificial induction of polyploidy can be achieved provides an opportunity for using this naturally-occurring phenomenon as a valuable tool in plant breeding programmes, where polyploidy has been used extensively as a tool for creating novelty in ornamental crops (Levin, 1983; Väinölä, 2000; Ascough et al., 2008). In general, tetraploids have larger flowers and fruit than their diploid counterparts and furthermore, because of their altered blooming periods, may have wider harvesting and marketing windows (Levin, 1983). These factors are particularly important in ornamental plants and cut flowers, when the potential for commercialisation is addressed. The artificial induction of polyploidy has been reported for a number of South African iridaceous genera, including *Watsonia* (Ascough et al., 2007, 2008) and *Gladiolus* (Suzuki et al., 2005).

Because naturally-occurring polyploid genotypes are usually unavailable, polyploidy is typically induced in breeding programmes

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through mitotic spindle inhibition or microtubule polymerization, often by exposure to colchicine (Caperta et al., 2006). Polyploid induction depends on the concentration of colchicine, the duration of exposure, explant type, and tissue penetrability (Allum et al., 2007). Colchicine-induced polyploidy is characterized by low induction rates and a high frequency of chimaeras or mixoploids which must be screened out of the population; this is most commonly achieved through flow cytometry analysis (Galbraith et al., 1997). If colchicine-induced pure tetraploids are not produced, rapid *in vitro* proliferation can be used to segregate pure tetraploids from chimaeras, due to the nature of the *in vitro* proliferation system.

Micropropagation has increasingly become a valuable tool for breeders, assisting in releasing new selections and cultivars into the market more rapidly. Ascough et al. (2009) reported that the first published record of *Crocosmia* micropropagation was by Koh et al., 2007. Ovaries and florets of *Crocosmia crocosmiiflora* were cultured with 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), 6-benzylaminopurine (BAP) or kinetin. Callus formed on ovaries at low frequencies (1–28%) with BA and 2,4-D, but rooting was prolific with either NAA or 2,4-D. When florets were used as starting material, roots and corms were induced using a combination of kinetin with either 2,4-D or NAA. These corms produced shoots when kinetin was applied, callus when 2,4-D was used in combination with BA, and corms when kinetin was used in combination with 2,4-D.

The primary objective of this study was to develop a rapid and efficient protocol for the micropropagation of *C. aurea*, to establish methods for polyploid induction and to assess selected floral characteristics of the resulting polyploid plants, with a view to the development and improvement of the species.

2. Materials and methods

2.1. Development of micropropagation protocols

Diploid seeds (obtained from Silverhill Seeds, Cape Town) of *C. aurea* were used as starting material to develop a micropropagation protocol. Five hundred seeds were sterilised for 20 min using a 1% [w/v] calcium hypochlorite solution. The sterilised seeds were rinsed three times with sterile distilled water before being cultured on standard MS medium (Murashige and Skoog, 1962 [MS]) containing 30 g l^{-1} sucrose, adjusted to pH 5.7 and sterilized by autoclaving for 20 min at 121 °C at 1 bar. Each seed was germinated in a glass tube containing 10 ml medium.

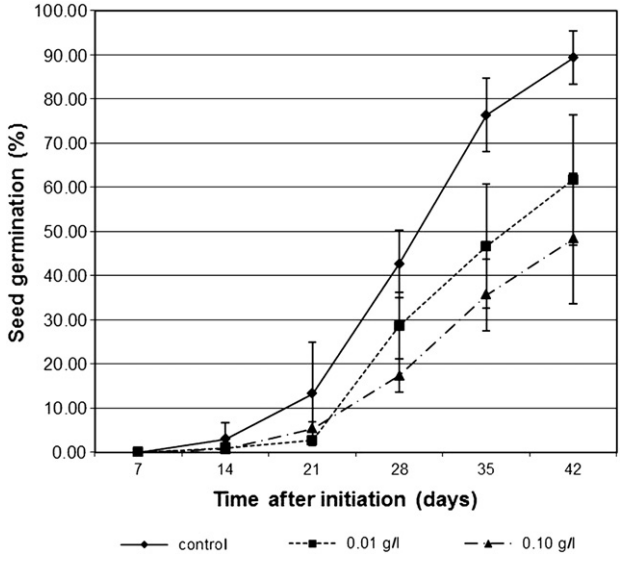

Fig. 1. Effect of colchicine concentration on seed germination of *Crocosmia aurea*.

The germinated seedlings were then transferred to MS medium containing one of four concentrations of BAP – 0.0 μM, 4.4 μM, 8.8 μM or 13.2 μM – to determine the most appropriate medium for plantlet multiplication. Each of the four BAP treatments comprised three replicates with 20 plantlets per replicate. Plantlets were maintained at 25 to 27 °C under a 16/8 h light/dark regime with cool white fluorescent light (81 μmol m^{-2} s^{-1}, Phillips 65 W) and were subcultured every 4 to 6 weeks. Once multiplied and rooted, the medium was rinsed from the roots and plantlets were hardened-off for 4 weeks in a mist bed (housed within a polycarbonate tunnel maintained at 15 to 27 °C) in Speedling® trays containing a 1:1 (v/v) mixture of composted pine bark and coarse river sand, before being planted out into 2 l potting bags containing a 1:1 (v/v) mixture of composted pine bark and sand. Plants were thereafter maintained in a shade house (40% shade cloth) at ambient conditions. Plants were watered daily on an irrigation system and Osmocote® was applied as a slow-release fertiliser.

2.2. In vitro induction of polyploidy

Diploid seeds of *C. aurea* were used as starting material to produce tetraploid plants, with the protocol described above being used to multiply plantlets generated from the treated seed. Five hundred diploid seeds were physically scarified to facilitate colchicine uptake and then treated, under aseptic conditions, with a 25 μM sterile colchicine solution for 3 days or, alternatively, a 0.25 μM solution overnight (based upon results previously obtained in our laboratory). The treated seeds were cultured on the previously determined optimum *in vitro* medium (MS medium supplemented with 4.4 μM BAP). Germination rate was recorded over 6 weeks, as was the final germination percentage. Proliferation rate (number of shoots per 4–6 week subculture interval), shoot height and rooting percentage were measured to determine differences between the two colchicine treatments as well as between diploid and tetraploid shoot cultures. Plants were maintained *in vitro* until they were large enough for ploidy analysis (approximately 12 weeks after treatment) and subculture. The experiment was repeated three times.

2.3. Ploidy analysis and proliferation of confirmed tetraploid plants

The ploidy level of treated seedlings was verified using a Partec PA ploidy analyser (Partec, Germany). Samples were prepared for flow cytometry analysis using approximately 1 cm^2 of leaf tissue. The tissue was macerated with a razor blade in 125 μl of nucleus extraction solution (Partec, Germany), after which the homogenate was filtered through a 50 μm mesh filter. The isolated nuclei were stained with 1250 μl 4′-6-diamidino-2-phenylindole (DAPI) stain (Partec, Germany) prior to commencing flow cytometry analysis. Nuclei isolated from untreated, diploid plantlets were used as a standard. Histograms were analysed using the Partec software package. Seedlings confirmed as tetraploid were proliferated on MS medium containing 4.4 μM BAP under the same conditions described above. Plantlets were hardened-off as described above and used to determine polyploidy effects on various horticultural characteristics.

2.4. Morphological characterisation of tetraploids

In order to evaluate whether polyploidy induction had an effect on the horticultural characteristics of *Crocosmia*, diploid and tetraploid plantlets were proliferated and maintained as described above. The following characteristics were evaluated once the micropropagated plants had hardened-off and reached maturity: leaf width and length, flower diameter, petal width and length, stigma, stamen and anther length, inflorescence diameter and length, as well as flower bud number per inflorescence.