*Plant Cell, Tissue and Organ Culture* **43:** 51–57, 1995.



# Adventitious *in vitro* plantlet formation from immature floral stems of *Crinum macowanii*

M.M. Slabbert[1*], M.H. de Bruyn[1], D.I. Ferreira[1] & J. Pretorius[2]
[1] *Vegetable and Ornamental Plant Institute, Agricultural Research Council, Private Bag X293, Pretoria, 0001, Republic of South Africa;* [2] *Department Botany, Rand Afrikaans University, P.O. Box 524, Johannesburg, 2000, Republic of South Africa*

Received 15 June 1993; accepted in revised form 28 June 1995

*Key words:* Bulbs, bush lily, histological study, regeneration, shoots

## Abstract

Adventitious shoots and plantlets were regenerated *in vitro* from floral stem explants of *Crinum macowanii* (Bak.) (bush lily). The length (age) of the floral stem as well as the orientation and position of the explant disc in the floral stem were the most important factors affecting shoot regeneration. The highest number of shoots were regenerated when immature floral stems of 70–100 mm were used as starting material, using the middle or basal parts of the stem, and orientating the discs with their proximal ends on the medium. Combinations of kinetin (4.65 $\mu$M) and either indoleacetic acid (0.57 $\mu$M) or naphthaleneacetic acid (0.54 $\mu$M), or a combination of benzyladenine (4.44 $\mu$M) and 2,4-dichlorophenoxyacetic acid (0.45 $\mu$M) resulted in the highest numbers of shoots being regenerated. Although a slight degree of callus formation was noticed on the cut-edges of the discs, shoot formation did not occur via callus, but directly from the floral stem epidermis. Unrooted shoots were rooted on MS-medium containing 0.17 M sucrose.

*Abbreviations:* BA – benzyladenine; IAA – indoleacetic acid; IBA – indolebutyric acid; NAA – naphthaleneacetic acid; 2,4-D – 2,4 dichlorophenoxyacetic acid

## Introduction

Amaryllidaceae is of horticultural importance and this, together with the threatened existence of the bush lilies (Slabbert *et al.* 1993), resulted in the decision to apply tissue culture techniques to multiply these bulbous plants. The regeneration of bulblets from twin scales of *Crinum macowanii* (Bak.) *in vitro* has been reported by Slabbert *et al.* (1993). The present report deals with the *in vitro* regeneration of bulblets from flower stems. It has been demonstrated by various workers that shoots and plantlets can be regenerated from floral stems of the Amaryllidaceae, for example in *Hippeastrum* (Hussey 1975), *Amaryllis belladonna* (de Bruyn *et al.* 1992), *Ipheion* (Hussey 1975) and *Nerine* (Pierik & Steegmans 1986). The potential advantages of using floral stem explants of *Crinum macowanii* are discussed, as well as the effects of size and orientation of explants, length (age) of flower stems and growth regulators on regeneration.

## Materials and methods

Flowering bulbs (approx. 50–60 mm diam.) of *Crinum macowanii* were harvested from the field during the beginning of the flowering period of November to January. Bulbs were disinfested as described by Slabbert *et al.* (1993), after which the bulbs were cut vertically to remove the one to three floral stems present in each bulb. Bulbs that were due to flower were harvested periodically in order to obtain floral stems of different lengths (50–160 mm). Floral stems (1 to 3 per bulb) were removed as soon as the tip of the first floral stem was visible above the bulb scales. Aseptic floral stems were cut into discs.

*Growth regulators*

The basic inorganic salts and sucrose of Murashige & Skoog (MS) -medium (1962) as modified by De Fossard (1985) were used (control) as well as treatments containing BA (4.44 $\mu$M) or kinetin (4.65 $\mu$M) in combination with either IAA (0.57 $\mu$M), IBA (0.49 $\mu$M), NAA (0.54 $\mu$M) or 2,4-D (0.45 $\mu$M). The pH of the medium was adjusted to 5.7 whereafter 0.18% gellan gum (Gelrite ™) was added to the medium before autoclaving (120 °C, 20 min). The floral stems (50–100 mm length) were cut into 5 mm thick disks and divided at random over the series of regeneration media. Explants were cultured in specimen tubes (22 mm diameter, 100 mm height, aluminium caps) each containing 10 ml nutrient medium. Half of the cultures was placed in a 16/8 h light/dark regime at a photosynthetic photon flux (PPF) of 12.5 $\mu$mol $m^{-2}$ $s^{-1}$ cool white fluorescent light and 24 ±1 °C for 64 days, while the other half was first incubated in the dark for 50 days, whereafter tubes were placed for a further 14 days with the former cultures in a 16/8 h light/dark regime.

*Rooting*

Shoots that were initiated from floral stem disks, were rooted on MS-medium containing sucrose concentrations of 0, 0.03, 0.06, 0.09, 0.17 or 0.26 M. Rooted plantlets were planted in peat:bark (2:1) in seedling trays and maintained in a glasshouse with a day temperature of 27–30 °C, a night temperature of 10–15 °C, and PPF of 500 $\mu$mol $m^{-2}$ $s^{-1}$. Commercial plant nutrition (Multifeed™) was given every 3 weeks. After a period of 2 years the plants were transferred to beds [peat:bark:soil (1:1:1)] in a shade house (30% shade).

*Orientation, size and length of floral stems*

Aseptic floral stems were cut into discs 2.5–5.0 mm thick. Floral stem explants were divided at random and placed either adaxially or abaxially on the MS-medium containing 4.65 $\mu$M kinetin and 0.54 $\mu$M NAA. Regeneration from four floral stems of different lengths (A = 70 mm , B = 100 mm, C = 130 mm, D = 160 mm) were also compared *in vitro*. After a period of approximately 90–100 days in culture (16/8 h light/dark), shoots and plantlets formed were scored according to size or number, while factors such as callus formation and general vigour of the shoots and plantlets were noted.

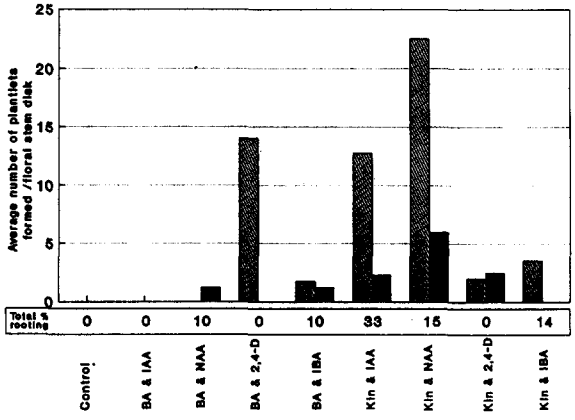


*Fig. 1.* The effect of i) BA (4.44 $\mu$M) and kinetin (4.65 $\mu$M) in combination with IAA (0.57 $\mu$M), IBA (0.49 $\mu$M), NAA (0.54 $\mu$M) & 2,4-D (0.45 $\mu$M) and ii) 16/8 h light/dark for 64 days ▨ or 50 days dark, then 16/8 h light/dark for 14 days ■ (N=10 for all treatments) on the regeneration of shoots from floral stem disks of *Crinum macowanii in vitro*.

*Origin of shoots*

In order to determine the origin and development of the shoots from the floral stem explants, the explants were studied histologically. Samples of floral stem discs were placed on MS-medium containing 4.65 $\mu$M kinetin and 0.54 $\mu$M NAA. Explants were fixed in Craf (Sass 1966), dehydrated in a series of alcohol and xylene whereafter they were embedded in Histosec™ wax. Serial longitudinal sections of 10–12 $\mu$m were cut and stained with safranin and fast green.

*Statistical analysis of data*

Significant differences were calculated using the Chi-square test ($p \leq 0.05$) and differences are denoted in figures by different letters.

## Results

*Growth regulators*

After only 10 days on media, the floral stem discs extended to nearly double their size in diameter. More shoots and plantlets were formed from floral stem discs incubated at 16/8 h light/dark than from discs first incubated in the dark (Fig. 1), although the shoots that formed from the latter explants, which were first incubated in the dark, were more vigorous. The largest number of shoots per explant were formed on MS-

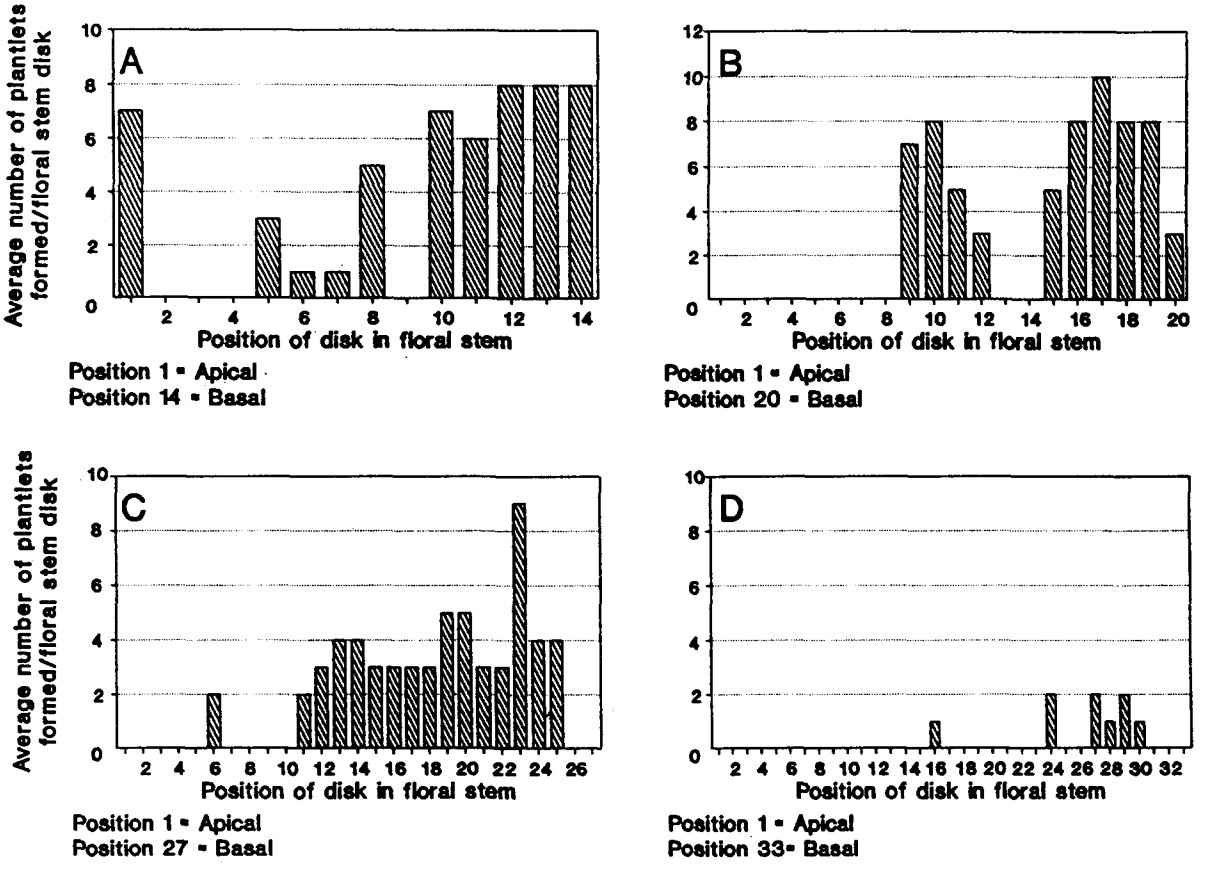


*Fig. 2.* The effect of length (age) of four floral stems (*A*=70 mm; *B*=100 mm; *C*=130 mm; *D*=160mm) on the initiation of shoots of *Crinum macowanii in vitro* after a period of 90–100 days in culture.

media containing 4.44 $\mu$M BA and 0.45 $\mu$M 2,4-D 4.65 $\mu$M kinetin and 0.57 $\mu$M IAA and 4.65 $\mu$M kinetin with 0.54 $\mu$M NAA in the 16/8 h light/dark, while no plantlets were formed on the control and 4.44 $\mu$M BA with 0.57 $\mu$M IAA. A higher percentage of explants forming callus on the cut edges of the discs was noted on treatments with 4.65 $\mu$M kinetin and 0.54 $\mu$M NAA as well as 4.65 $\mu$M kinetin and 0.45 $\mu$M 2,4-D. A low percentage of rooting occurred on some of the treatments (Fig.1).

*Orientation, size and length of floral stems*

Explants from the apical regions of the floral stem showed no totipotency, and these explants became brown and died. After a period of 64 days, significantly more shoots ($p \leq 0.05$) were formed on the morphologically apical edges of the discs (regardless of the orientation of the discs) (Table 1) with the second highest number of shoots formed on the morphologically basal edges and from the epidermis, while fewer shoots formed on the cut surfaces of the discs. Although the average number of shoots formed per explant was higher on the 5.0 mm thick discs, the difference between the number of shoots formed on 2.5 and 5.0 mm discs was not statistically significant. The middle and basal parts of the floral stems were statistically more regenerative than the apical parts ($p \leq 0.05$). Floral stems of 130 mm or longer showed little or no regenerative ability (Fig. 2).

*Rooting*

After a period of 6 weeks the highest percentage of rooting occured on MS-medium containing 0.17 M sucrose (Fig. 3). A 100% survival rate was obtained when rooted plants were planted in the glasshouse and transferred to a shade house.

*Table 1.* The effect of the orientation of four different floral stem discs (A-D) on media on the *in vitro* regeneration of shoots from *Crinum macowanii* after 90–100 days in culture.

| Floral stems A-D/ Orientation | Average number of shoots formed per floral stem disc | | | |
|---|---|---|---|---|
| | Apical edge of floral stem disc | Basal edge of floral stem disc | Epidermis of floral stem disc | Cut surface of floral stem disc |
| A (N=24) ↓*b* | 2.7 | 1.8 | 0.5 | 0.4 |
| B (N=27) ↓*b* | 1.3 | 0.2 | 0.2 | 0.5 |
| C (N=20) ↓*a* | 1.8 | 0.6 | 1.4 | 0 |
| D (N=33) ↓*a* | 0.13 | 0.06 | 0.1 | 0 |
| Average of A-D | 5.4*a | 2.7*b | 2.2*b | 0.9*c |

↓*b* Floral discs with proximal ends on medium
↓*a* Floral discs with distal ends on medium
* Different letters show statistically significant differences ($p \leq 0.05$)

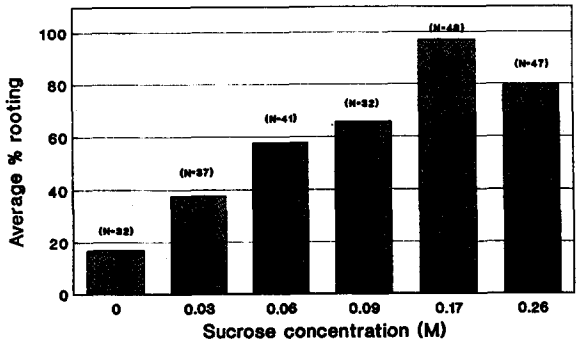


*Fig. 3.* Rooting of plantlets initiated from floral stem disks on MS-medium with different sucrose concentrations after a period of 6 weeks.

### *Origin of shoots*

Shoot formation was not preceded by callus formation, and shoots developed adventitiously from the floral stem epidermis. Only a few shoots developed on the cut surface of the floral stem. After a period of 35 days, anticlinal divisions of the epidermis were noticed (Fig. 4a) and a bud primordium was formed in the epidermis and sub-epidermal layers as a result of the periclinal divisions of the hypodermis after 40 days (Fig. 4b). After a period of approximately 45 days further divisions of the epidermis and hypodermis gave rise to the development of a bud primordium that had already begun differentiating an apical meristem that was macroscopically visible (Fig. 4c). An apical meristem with two leaf primordia was visible after a period of approximately 50–60 days (Fig. 4d).

## Discussion

In contrast to the floral stems of *Nerine* (Pierik & Steegmans 1986) and *Hippeastrum* (Pierik *et al.* 1990), which had to be cultured in darkness to obtain regeneration, more shoots were regenerated from the floral stem explants of *Crinum macowanii* kept at a con-

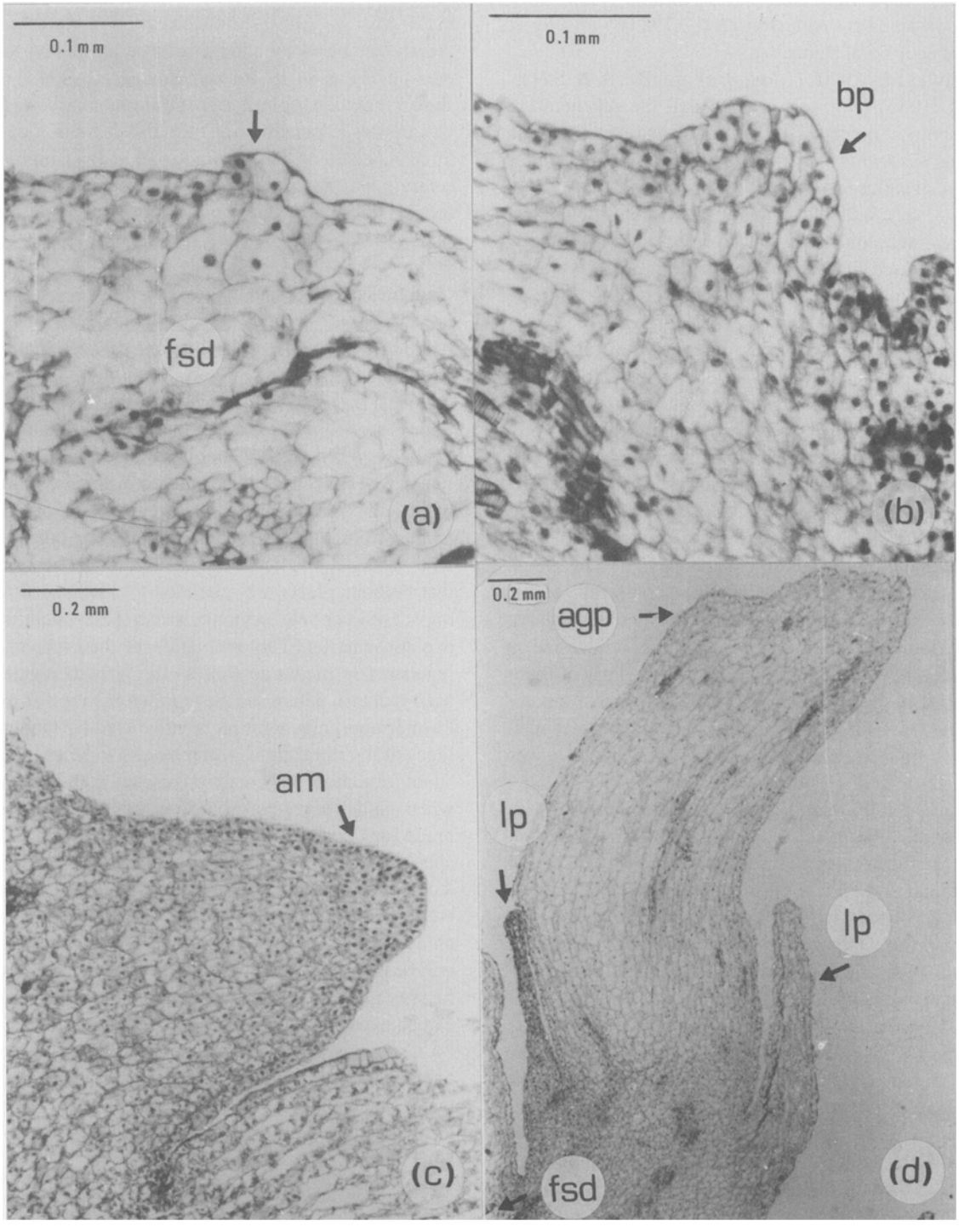


*Fig. 4. a-d* : The development of shoots from flower stems of *Crinum macowanii in vitro*. *(a)* Longitudinal section of floral stem disc (fsd) showing periclinal division of the outer cell layers (↑) 30–35 days after incubation. *(b)* Periclinal and anticlinal divisions of the epidermal and subepidermal cell layers resulting in a bud primordium (bp) after 35-40 days. *(c)* Continuation of these periclinal and anticlinal divisions developing an apical meristem (am) after 40-50 days. *(d)* Longitudinal section of floral stem disc (fsd) showing an adventitious growth point (agp) with 2 leaf primordia (lp).

tinuous 16/8 h light/dark period. Similar results have been obtained by Alderson *et al.* (1983) with *Tulipa* (Liliaceae) floral stems.

Ziv *et al.* (1970) (*Gladiolus*) and Pierik & Steegmans (1986) (*Nerine*) reported that the regeneration of shoots is preceded by callus formation. This was not the case with *Crinum macowanii* although a slight degree of callus formation was noticed on the cut edges. Shoots were initiated adventitiously from the epidermis and sub-epidermal cell layers of the floral stems, which caused the bulging of the peripheral tissue, as was also found by Hosoki & Asahira (1980) in *Narcissus* and Wright & Alderson (1980) in *Tulipa*.

It appears that the orientation of the floral stem explants plays an important role in the initiation of shoots from these explants. Most shoots were formed when discs were placed with their morphologically basal ends on the medium. This is in agreement with the results of work done on some species of the Liliaceae and Amaryllidaceae (Hussey 1975; Pierik & Steegmans 1986). The reason for this could be that most shoots developed on the apical edge of the discs. This had the implication that the shoots that developed from discs facing their apical sides downwards grew down into the medium, which may have led to the occurrence of hyperhydricity and the loss of these shoots. Shoots further developed to a lesser or greater degree, over the complete disc area, in contrast with floral stem discs of *Gladiolus* where the initiation was limited to the distal cut-edges (Ziv *et al.* 1970).

Research done by Wright & Alderson (1980) with *Tulipa* and Pierik & Steegmans (1986) with *Nerine* floral stems showed that more shoots developed from the apical and middle parts of the floral stem. This is again in contrast with the results obtained in the present study on floral stems of *Crinum macowanii* where most shoots were initiated from the middle and basal parts of the floral stems. Shoot formation was strongly determined by the length (age) of the floral stems. Shorter stems of 70–130 mm long had the best regeneration potential, while regeneration potential declined in stems longer than 130 mm. According to Taeb & Alderson (1987) the variation in regeneration potential of the floral stems is due to the different developmental stages of the bulbs, as well as the difference in position of the discs between the different flower stems. This was also noticed by Pierik & Steegmans (1986) and no clear explanation could be given for this.

It has been observed that higher sucrose concentrations in the medium, in contrast with shoot induction, increase rooting of a number of species. In *Lilium* sucrose at 0.26 M increased root dry weight (Takayama & Misawa 1979). Root formation of *Episcia* was greatly enhanced by adding sucrose at 0.09 M to the medium (Pearson 1979). Hyndman *et al.* (1983) have shown that the absolute concentration of carbohydrate was in part responsible for root differentiation of cultured rose shoots. The promoting effect of high concentrations of sucrose was also demonstrated on the rooting of *Crinum macowanii* shoots.

## Conclusion

Although the *vitro* multiplication of the Amaryllidaceae by means of twin scales is well documented, it is clear from these results that the floral stems can also be used for the propagation of these bulbous plants. It is a relatively easy method to follow, and less contamination can be expected when using floral stems compared with twin scales. There are, however, also limitations to this technique. A fact that should be considered when using floral stems for *in vitro* multiplication, is that bulbous plants are restricted to only one flowering season per year, which restricts the availability of explant material. The orientation of the disks on the regeneration media as well as the growth regulators used will also determine the number of shoots formed. Furthermore, regeneration is influenced by the length (age) of the floral stem, which seems to be critical for shoot formation. Regeneration also takes twice as long when using floral stems than does the twin scale technique for *Crinum macowanii* (Slabbert *et al.* 1993). In spite of all the above mentioned factors, it is still a technique with potential, particularly since the regeneration rate from floral stems is relatively high (up to an average of 22 shoots per disc), and this technique will be particularly useful in instances where bulbs are in a poor condition and thus a high contamination rate can be expected when using twin scale explants.

*Crinum macowanii* plantlets, multiplied and established in this manner, adapted and survived 100% under shade house conditions. Rate of growth and time to flower were similar to that of seedlings of *Crinum macowanii*.

## Acknowledgements

Many thanks to Dr. F. Niederwieser for her help and support during this study. We also thank Mrs. M. Smith

of Biodatametrics for helping with the statistical analysis of the data.

## References

Alderson PG, Rice RD & Wright NA (1983) The potential for propagating tulips through tissue culture. The Plant Propagator 29 : 10–13

De Bruyn MH, Ferreira DI, Slabbert MM & Pretorius J (1992) *In vitro* propagation of *Amaryllis belladonna*. Plant Cell Tiss. Org. Cult. 31: 179–184

De Fossard RA (1985) Tissue culture propagation: state of art. Acta Hort.166: 83–91

Hosoki T & Asahira T (1980) *In vitro* propagation of *Narcissus*. HortScience 15: 602–603

Hussey G (1975) Totipotency in tissue explants and callus of some members of the Liliaceae, Iridaceae and Amaryllidaceae. J. Exp. Bot. 26: 253–262

Hyndman SE, Hasegewa PM & Bressan RA (1983) The rale of sucrose and nitrogen in adventitious root formation on cultured rose shoots. Plant Cell Tiss. Org. Cult. 2: 229–238

Murashige T & Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Pearson AE (1979) Effects of auxin and cytokinins on morphogenesis of three varieties of *Episcia* In: Conger BV (ed) Cloning Agricultural Plants Via *In Vitro* Techniques (pp 5–40). CRC Press, Boca Raton, Florida

Pierik RLM & Steegmans HHM (1986) Adventitious plantlet regeneration from floral stem explants of *Nerine bowdenii* W Watts. Ned. J. Agr. Sci. 34: 217–223

Pierik RLM, Blokker JS, Dekker MWC, De Does H, Kuip AC, Van der Made TA, Menten YMJ & De Vetten NCMH (1990) Micropropagation of *Hippeastrum* hybrids. Integration of in *vitro* techniques in ornamental plant breeding. In: de Jong J (ed) Integration of *In Vitro* Techniques in Ornamental Plant Breeding. Proceedings, Symposium. 10–14 November 1990 (pp 21–27), Eucarpia, Wageningen, Netherlands

Sass JE (1966) Botanical Microtechnique. Iowa State Univ. Press (183 p)

Slabbert MM, De Bruyn MH, Ferreira DI & Pretorius J (1993) Regeneration of bulblets from twin scales of *Crinum macowanii in vitro*. Plant Cell Tiss. Org. Cult. 33 : 133–141

Taeb AG & Alderson PG (1987) Micropropagation of tulip: optimising shoot production from floral stem explants. Acta Hort. 212: 677–681

Takayama S & Misawa M (1979) Differentiation in *Lilium* bulbscales grown *in vitro* - effect of various cultural conditions. Physiol. Plant. 46: 184–190

Wright NA & Alderson PG (1980) The growth of tulip tissues *in vitro*. Acta Hort. 109: 263–270

Ziv M, Halevy AH & Shilo R (1970) Organs and plantlets regeneration of *Gladiolus* through tissue culture. Ann. Bot. 34: 671–676