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Adventitious plantlet regeneration from floral stem explants of *Nerine bowdenii* W.Watts.[1]

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Abstract

Adventitious bulblet regeneration from floral stem explants of *Nerine bowdenii* W.Watts. was studied in vitro at 18 °C in continuous darkness. Explants, 1.5 mm thick, were cultured with basal ends down on a basic medium with BA 1 mg/litre and NAA 0.5 mg/litre. Optimal bulblet regeneration occurred when immature floral stems, 2.5 to 10.0 cm in length, were taken.

To obtain plantlets the regenerated bulblets or explants with bulblets had to be subcultured on a cytokinin-free medium in continuous light at 25 °C. Plantlets could easily be transplanted to soil.

Introduction

Since the propagation rate of *Nerine* species through natural offsets is relatively low, twin scaling has been introduced. Pierik & Ippel (1977) developed an in vitro method to increase the propagation rate and described plantlet regeneration from excised bulb scale segments. Grootaarts et al. (1981) demonstrated that in *Nerine bowdenii* the regeneration of adventitious bulblets always occurred at places where the scales contain basal plate tissue.

Subsequently the question arose whether it was also possible to induce adventitious bulblets on other organs than the bulb scales. Since there are many reports on the regeneration of adventitious sprouts and bulblets on explants of (young) inflorescences and floral stem explants, an attempt was made to induce adventitious bulblet formation in explants of floral stems of *Nerine bowdenii*. This paper presents the results of the investigations.

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Materials and methods

Bulbs of *Nerine bowdenii* W.Watts. cv. 'Favoriet' served as starting material. Dry bulbs first received a cold treatment (5 °C) for at least 4 months to break dormancy before planting in a greenhouse at 14-16 °C. Flowering plants were periodically harvested to obtain floral stems of various lengths (1 to 20 cm).

Floral stems were sterilized by immersion in 96 % ethanol for a few seconds, rinsing in 10 % commercial bleaching liquor (containing 10 % NaOCl) with a few drops of Tween 20 for 20 minutes, and finally rinsing 3 times in sterilized tap water for 30 minutes.

Sterilized floral stems (usually 2.5 to 4.0 cm long and 0.5 cm in diameter) were subsequently cut into 1.5 mm thick disks (slices) which were placed with their basal ends down on the culture medium. In most experiments disks were divided at random over the various treatments; in so-called gradient experiments the original position of the explants in the stem was recorded.

Unless stated otherwise, the basic medium contained: MS (Murashige & Skoog, 1962) macro-elements at half strength, MS (Murashige & Skoog, 1962) micro-elements (except Fe), NaFeEDTA 25 mg/litre, vitamin B_1 0.4 mg/litre, méso-inositol 100 mg/litre, sucrose 4%, BA (6-benzylaminopurine) 1 mg/litre, the potassium salt of NAA (1-naphthaleneacetic acid) 0.5 mg/litre, Difco-Bacto agar 0.7 %. The pH was adjusted to 6.0 before autoclaving. Explants were cultured in Pyrex glass test tubes, each containing 15 ml of medium.

Generally adventitious bulblet regeneration was studied at 18 °C in continuous darkness. The effect of temperature was examined in airconditioned growth rooms (13-25 °C). When the cultures were transferred to light after 10 weeks of darkness, they were kept in continuous fluorescent light (Philips TL40/57, 7 W/m^2). Rooting and sprouting of subcultured bulblets or explants took place at 25 °C under the light conditions described above.

Bulblet regeneration was periodically scored, whereas final number and weight of the bulblets per explant were determined after 10 weeks continuous darkness. Mean number of bulblets per floral stem explant were calculated over all excised explants (with or without regeneration) except the infected ones. Each treatment consisted of 24 explants. Preliminary experiments were carried out in 1982 and repeated under optimized conditions in 1983.

The experimental set-up was that in each experiment only 1 to 2 plant or environmental factor(s) was(were) varied, while all other factors were kept constant.

Results

Bulblet regeneration

Preliminary experiments were carried out in 1982 to study various plant and environmental factors; in this way it was possible to eliminate so-called limiting factors in the experiments in 1983. The standard conditions described in 'Materials and methods' can therefore be considered as optimal for bulblet regeneration: 18 °C,

Fig. 1. Floral stem explant of *Nerine bowdenii* with callus formation at the distal side. In the callus several adventitious bulblets are formed. Photo taken 10 weeks after isolation.

darkness, sucrose 4 %, BA 1 mg/litre, NAA 0.5 mg/litre, etc. When the sterilization procedures were followed, the infection rate was 5-10 %.

Bulblet regeneration was always preceded by callus formation on the cut surfaces of the explants, particularly at the distal sides on which most bulblets were formed (Fig. 1). Bulblet regeneration was promoted when floral stem explants were placed on the medium with their basal ends down as opposed to basal ends up. By increasing the diameter of the floral stems the number of bulblets was strongly increased; 52 % of the explants from a stem with a diameter of 4 mm regenerated with an average number of bulblets of 1.5, whereas 96 % of the stems with a diameter of 8 mm regenerated with a mean number of bulblets of 7.1. Averaged over all experiments, standard explants (1.5 mm thick taken from stems with a length of 2.5-4 cm and a diameter of 5 mm) regenerated for 72 % with an average number of bulblets of 2.8 per explant; for unknown reasons some explants always browned and did not regenerate.

Bulblet regeneration was strongly determined by the age (length) of the original floral stem. Very young stems (0.5 cm in length) hardly regenerated, young stems (1 to 1.5 cm) regenerated moderately, whereas the best regeneration was obtained from stems with a length of 2.5 to 5.0 cm. As stems become longer, regeneration decreased and completely stopped at a stem length of 20 cm or more. A comparison of basal, middle and top explants taken from 2.4 to 4 cm long stems demonstrated that regeneration in middle and top explants was more or less equal, whereas regeneration in basal explants was less than in middle and top explants.

In the temperature range 13 to 25 °C, 18 °C appeared to be the optimum for bulblet regeneration, whereas at 21 °C bulblet regeneration decreased; at 25 °C the regeneration rate was very low and much browning occurred. The influence of light was only examined by culturing the explants either in continuous light or continuous darkness; all experiments showed that adventitious bulblet formation was better in darkness than in light.

The presence of sugar in the medium appeared to be very important; sucrose was

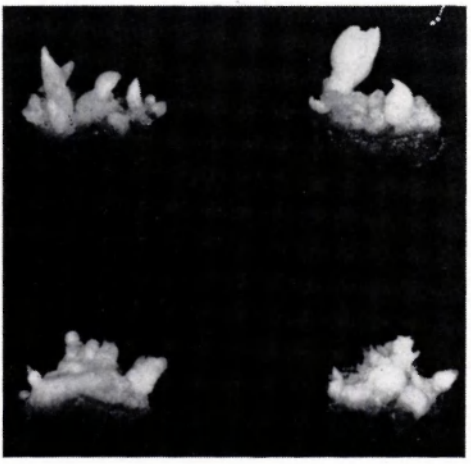

Fig. 2. Adventitious bulblet regeneration on floral stem explants as affected by the BA concentration. Upper row: outgrowth of the bulblet primordia on a medium with BA 1 mg/litre and NAA 0.5 mg/litre. Lower row: the outgrowth of bulblet primordia is inhibited on a medium with BA 5 mg/litre and NAA 0.5 mg/litre.

slightly more effective than glucose. In the sucrose concentration range from 1 to 5 %, 4 % appeared to be the optimum. The effect of the concentrations of macro-elements was tested at 1/2, 3/4, and 1/1 (full) strength MS macro-elements; the highest regeneration percentage and number of bulblets were observed at half-strength MS, whereas regeneration parameters decreased by increasing the MS concentration from 1/2 to 1/1 strength.

The presence of both auxin and cytokinin in the medium was essential, because no callus formation and regeneration took place without auxin and/or cytokinin. Explants were incubated on media with 0.5, 0.8, 1.0, 3.0, or 5.0 mg/litre BA, each in combination with NAA at 0.2, 0.5, or 0.7 mg/litre. The combination of BA 1.0 mg/litre with NAA 0.5 mg/litre was found to be the optimum for adventitious bulblet formation (Fig. 2).

Subculture to obtain plantlets

To obtain plantlets from the regenerated bulblets, the following procedure was optimal. Regenerated bulblets or explants with bulblets had to be subcultured in continuous light at 25 °C; a temperature of 25 °C was much better for plantlet formation than 18 or 21 °C, whereas light was essential for sprouting. The culture medium had to be changed as follows: MS macro-elements full strength was used instead of half strength (Fig. 3), whereas the cytokinin BA was omitted. A NAA concentration of 0.5 mg/litre appeared to be favourable for plantlet formation.

Subculture of bulblets without floral stem tissue was only advisable when the bulblets, that were formed on the floral stem explants, had a diameter of at least 3-5 mm; when smaller bulblets were isolated without floral stem tissue, they did not produce plantlets or even died.

The only way to obtain also plantlets from the bulblets smaller than 3 mm is to subculture bulblets connected with floral stem tissue. Stem explants with a large diameter can be cut into two halves, which then are separately subcultured; ex-

plants with a small diameter should be left intact when subcultured. To promote plantlet formation from the small bulbs on the stem explants, brownish tissue at the bases of the explants should be removed. Eight weeks after the start of subculture,

Fig. 3. The influence of the MS concentration during subculturing of floral stem explants with regenerated bulblets. Upper row: poor growth on MS half strength. Lower row: good growth on MS full strength.

Fig. 4. Plantlets formed after 8 weeks of subculture at 25 °C. Upper row: floral explants on which bulblets form leaves and roots. Lower row: subculture of isolated bulblets which also form leaves and roots.

plantlets (Fig. 4) had developed to such a size in vitro (success rate about 75 %) that they could be transplanted to soil. Plants were grown in the greenhouse (plastic shelter), where growth rapidly continued. Whether the in vitro produced plants were true to type is not yet known, since flowering has not yet occurred.

Discussion

Most genera of the Amaryllidaceae can only regenerate bulblets at the basal sides of the scales or leaves, especially in those tissues close to the basal plate: *Narcissus* (Hussey et al., 1978), *Nerine* (Pierik & Ippel, 1977; Grootaarts et al., 1981), *Hippeastrum* (Yanagawa & Sakanishi, 1980), *Eucharis* (Pierik et al., 1983). For *Narcissus* there are indications that flower parts are also capable of regenerating (Hussey, 1982). Our results demonstrate that totipotency in *Nerine bowdenii* is not restricted to tissues near the basal plate; floral stem tissues can also be transformed into meristematic cells giving rise to bulblets of adventitious origin. Since it was recently found that flower stalk explants of *Nerine sarniensis* 'Corusca Major' and *Eucharis amazonica* (Pierik, Sprenkels & Steegmans, unpublished) are capable of regenerating adventitious bulblets, it is supposed that floral stems are likely to be an excellent source for in vitro propagation of most Amaryllidaceae.

Adventitious bulblet formation in floral stem explants of *Nerine bowdenii* appears to be influenced by a complex of plant, environmental and hormonal factors. When certain requirements are not met, bulblet regeneration is strongly reduced or does not occur. The principle of limiting factors, both quantitative and qualitative, is therefore certainly applicable in the case of *Nerine*. To achieve optimal bulblet formation, we must know how to balance the many factors referred to above.

Our results with *Nerine* show a certain similarity with those obtained by Alderson et al. (1983) with floral explants of tulip. However, in contrast to tulip, floral explants of *Nerine* appeared to be very sensitive to light and should therefore be cultured in darkness to obtain bulblets; another characteristic difference between tulip and *Nerine* was, that tulip almost completely lost the ability to regenerate when the dry bulbs were planted and entered the active growth period (root and leaf formation, and floral stalk elongation), whereas for *Nerine* active growth was essential. Bulblet formation in *Nerine* appeared to occur easily, but in tulip it could only be induced by cold treatment or exposure to gibberellins.

Conclusions

The vegetative propagation of *Nerine bowdenii* through floral stem explants can be divided into three phases: (1) the regeneration of adventitious bulblet at 18 °C in continuous darkness, in the presence of cytokinin and auxin; (2) the formation of shoots and roots at 25 °C in light with only auxin; (3) the transfer from test tube to soil. Since bulblets were regenerated adventitiously from callus on the cut surfaces of the floral explants, the possibility of mutations cannot be excluded. Since in vitro produced *Nerine bowdenii* plants have not yet flowered, it is impossible to state whether the plants produced through flower stem explants will be true to type.

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