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Influence of Temperature and Growth Regulators on Anther and Pollen Development During *In Vitro* Flowering of *Narcissus triandrus*

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ABSTRACT

During the *in vitro* propagation of *Narcissus triandrus* the culture conditions for the development of flowers with anthers and pollen comparable to those found *in vivo* were determined. Flowering was achieved, after 12 weeks of culture, from twin-scales of bulbs initially cultured on a modified Murashige and Skoog medium supplemented with benzyladenine (4 mg l^{-1} BA) and naphthaleneacetic acid (0.12 mg l^{-1} NAA) or with 2 mg l^{-1} BA and indolebutyric acid (1 mg l^{-1} IBA), with 3% sucrose, under constant 25ºC. Both media were adequate for either foliar primordia or floral bud induction; however, flower organs appeared disorganized both in disposition and structure. In the anthers, a typical callose wall surrounded pollen mother cells but the tapetum had already degenerated and most of the pollen grains had collapsed with a disordered exinic wall. When the cultures were transferred to medium containing only NAA and when sucrose was increased from 3% to 9% and maintained at 25ºC, a slight elongation of floral stalks was observed but the further development of flowers failed in 100% of plants. In contrast, at 18ºC, flowers morphologically identical to those found *in vivo* developed, displaying a well-organized tapetal layer and pollen grains with a correctly architectured wall. However, the lower temperature (18ºC) did not promote the development of normal flowers when cultures were maintained on induction media with BA and auxins. These results suggest that in this *Narcissus* species it is possible to induce flowering *in vitro* albeit conditioned by the composition of the culture media and strongly temperature-dependent.

Keywords: anther histology, BA, IBA, NAA, pollen ultrastructure

INTRODUCTION

The natural propagation of *Narcissus* by bulb offsets has a low multiplication rate per year. In addition to that, some *Narcissus* species are becoming threatened due to uncontrolled harvesting for local and international markets or due to habitat reduction by human encroachment. To overcome the eradication of these wild species, and as micropropagation techniques are a very powerful alternative to achieve a high number of new plants, the culture conditions for *in vitro* production of these bulbs were established at our laboratory (Santos I *et al*. 1998; Santos J *et al*. 1998; Santos and Salema 2000; Santos *et al*. 2002).

Besides its important role in plant propagation, plant tissue culture systems can be used to reduce significantly the juvenility stage of plants, allowing early flowering. *In vitro* flower production has been reported for several species (Taji *et al*. 2002; Taylor and van Staden 2006 and citations therein) and it is of utmost importance as it can provide a model system to study flower initiation and development and it can also be used to shorten the breeding period (Sudhakaran *et al.* 2006; Taylor and van Staden 2006).

During the *in vitro* propagation of *N. triandrus* from twin-scales (Santos I *et al*. 1998) the interaction between temperature and growth regulators on both shoot initiation and proliferation was analysed and the data obtained pointed to the importance of temperature under which the explants were cultured. Following this line of work, we undertook the study of *in vitro* flowering of *N. triandrus* and the influence of temperature and the culture media composition on anther and pollen development, that are here reported.

MATERIALS AND METHODS

Plant material and culture conditions

Bulbs were supplied by the Parque Natural da Serra da Estrela. They were collected from senescent flowering plants of *N. triandrus* grown in different locales of the referred mountains of central Portugal. These bulbs were cooled for 4 weeks (at 9ºC) and afterwards were used as a source of explants. To initiate the *in vitro* cultures, bulbs were carefully washed with detergent and rinsed in tap water. The sterilization was carried out in 80% ethanol (5 min) and in 20% sodium hypochlorite during 30 min and then with 8% sodium hypochlorite for 10 min. After several rinses with sterilized distilled water, the disinfected bulbs were cut as twin-scales and cultured on MS medium as modified by Hussey (1982). The basic medium with 3% sucrose was supplemented with 4 mg l^{-1} BA and 0.12 mg l^{-1} NAA (medium A) or with 2 mg l^{-1} BA and 1 mg l^{-1} IBA (medium B). All media were adjusted to a pH of 5.7-5.8 and then 0.6% agar was added before autoclaving at 121ºC (0.1 MPa) The time in the autoclave was varied according to the volume of medium in the vessel (17 min for 20 ml medium and 20 min for 60 ml medium). Cultures were maintained in growth chambers under an 11 h photoperiod and a photon flux density of 29 µmol m^{-2} s^{-1}. Two temperatures (25ºC and 18ºC) were tested during the culture period.

To initiate the cultures, about 12 healthy bulbs were selected. From each disinfected bulb the apical two-thirds was removed as well as the two external scales of the remaining portion; a thin layer of the basal plate was also discarded. The remaining portion of the bulb was longitudinally sectioned to obtain explants formed by segments of twin-scales, 2-3 mm wide and 8-10 mm high, joined by a thick segment (2-3 mm) of basal plate tissue. The explants were inoculated upright with the basal plate tissue

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inserted into the culture medium. From each bulb an average of 6-8 explants were obtained and for each culture medium, 30-40 explants were used. Cultures were initiated at 25ºC and, every six weeks, transferred onto fresh media. After the third period of culture, foliar shoots and flower buds, initiated at 25ºC, were transferred to the same basal medium supplemented with 0.12 mg l^{-1} NAA and 9% sucrose. These plants were divided into two sets, each set containing, at least, 40 plants. One set was maintained at 25ºC and the other in another growth chamber at 18ºC.

At each of the culture stages, flowers were excised and prepared for scanning microscope analysis and anthers for both optical and transmission electron microscopy.

Microscopic observations

Anthers were dissected and prepared for microscopy. Samples were fixed in 4% glutaraldehyde in 0.1 M Na_2-Na phosphate buffer, post-fixed in 2% osmium tetroxide in the same buffer, dehydrated with ethanol and embedded in Spurr's resin. Ultra-thin sections (60-80 nm thick) were contrasted with uranyl acetate followed by lead citrate and observed using a Zeiss EM C10 microspcope. Thin sections were stained with 1% methylene blue in 1% sodium tetraborate and 1% aqueous solution of Azur II (1:1) for optical microscope observation. Flowers fixed and dehydrated as described above were critical point dried, mounted on appropriate metal stubs, then sputter coated with gold and examined with a JEOL JSM-35C scanning electron microscope.

RESULTS

Following previous work concerning the establishment of culture conditions for the *in vitro* propagation of *Narcissus triandrus* (Santos I *et al.* 1998), we studied the ontogenic process of both anthers and pollen during *in vitro* flowering. During the initial period of culture, the response of twin-scale explants, under an incubation temperature of 25ºC, was similar on medium supplemented with 4 mg l^{-1} BA and 0.12 mg l^{-1} NAA (medium A) or with 2 mg l^{-1} BA and 1 mg l^{-1} IBA (medium B). Under these phytohormonal conditions, on both media after six weeks of culture, foliar shoot primordia emerged from the basal plate between the scales. After four to six weeks, these primordia developed into foliar shoots and afterwards into leaves; floral buds emerged from among them. Subsequent subculture onto fresh media and under the same temperature led to the elongation of floral stalks and the resultant development of flowers (**Fig. 1A**), but they differed in several aspects from field grown flowers. Unlike wild flower buds from field grown plants, which have two whorls of radial tepals sharply deflected and two whorls of three anthers each (Hanks 1993), all the flowers developed under these *in vitro* conditions displayed a disorganized distribution of the tepals and a high number of stamens (8-12 per flower), aspects clearly distinct in scanning electron microscope images (**Fig. 1B**). In the loculus of these anthers, cells at different ontogenic stages were observed; in some anthers, pollen mother cells surrounded by a callose wall could be observed but the characteristic tapetal layer was not discernable (**Fig. 1C**) while in other anthers, even of the same flower, cells had already attained the pollen grain stage, although most of the pollen collapsed and degenerated (**Fig. 1D**). One of the most striking disturbances, revealed by ultrastructural observations, occurred at the pollen grain wall. In comparison with the normal disposition in which a continuous exinic layer has columellae protruding from it, as observed in pollen grains of field grown plants (**Fig. 1E**), the exinic layers of the *in vitro* developed pollen displayed a disordered structure (**Fig. 1F**).

When on media A or B, the leaves developed tiny bulbs at their bases. The subsequent growth of foliar shoots plus bulbs was achieved on the same basal medium supplemented with 0.12 mg l^{-1} NAA and sucrose increased to 9%. After transfer to this medium, two sets were made with, at least 40 plants per set, one kept at 25ºC and the other at 18ºC. Interestingly, the behaviour of floral buds was markedly temperature-dependent. At 25ºC a slight elongation of floral stalks was observed, however 100% of the flowers aborted. On the contrary, under the lower temperature (18ºC), the developed flowers (**Fig. 1H**) were morphologically similar to those of *in vivo* grown plants. It is worthy to mention that the anthers of these *in vitro* flowers, at both early and late tetrad stages, displayed a well-organized tapetal layer (**Fig. 1I**); besides, ultrastructural studies showed that the exinic pollen wall (**Fig. 1G**) had an architecture morphologically identical to that of pollen grains of field grown plants (**Fig. 1E**). However, it should be emphasized that the beneficial effect of a lower temperature on the formation of normal flowers was only found when the culture medium contained NAA and 9% sucrose. In fact, if floral buds were transferred to 18ºC but maintained on the initial media, with BA and auxin (either media A or B), the development of normal flowers did not occur.

DISCUSSION

The results obtained for *in vitro* flowering of *N. triandrus* showed that this process is temperature-dependent. Besides, although cytokinins and auxins did not hinder the flower induction, the formation of normal flowers, morphologically similar to field grown ones, seems to be inhibited by cyto-

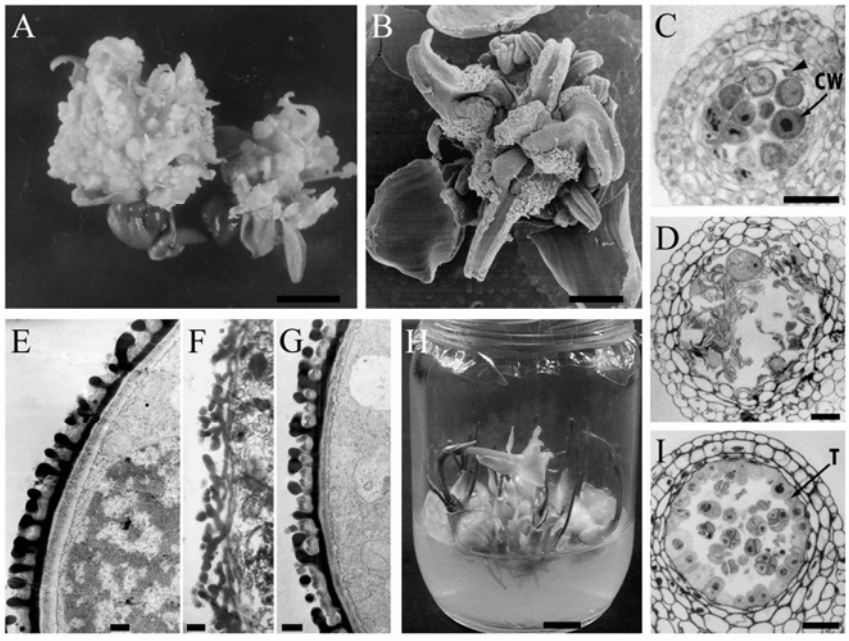

Fig. 1 Anther and pollen development during *in vitro* flowering of *Narcissus triandrus*. (**A**) Abnormal flowers of *N. triandrus* developed on media containing both BA and NAA at 25ºC. Bar = 1cm. (**B**) Scanning electron microscopy of abnormal flowers showing, in comparison with field grown flowers, a higher number of tepals and stamens with a disorganized distribution. Bar = 1 mm. (**C, D**) Cross-section of anthers of abnormal flowers. (**C**) Pollen mother cell stage. A callose wall surrounds pollen mother cells but the tapetal layer (arrowhead) is degenerated. (**D**) Pollen grain stage. Almost all the grains are shrunken and empty. Bars = 50 μm. (**E, F, G**) Ultrastructural aspects of pollen grain walls of pollen from field grown flowers (**E**), of pollen from abnormal flowers, with a disorganized exinic layer (**F**) and of pollen from normal flowers developed *in vitro* (**G**) with a structure similar to that of the control pollen. Bars = 0.5 μm. (**H**) Normal *in vitro* flowers developed on media containing NAA 0.12 mg l^{-1} and 9% sucrose under 18 ºC. Bar = 1 cm. (**I**) Cross section of an anther of a normal flower at late tetrad stage. Note the distinct tapetal layer (T) surrounding pollinic cells. Bar = 50 μm.

kinin in the medium.

Flowering involves a complex system of exogenous and endogenous factors such as photoperiod, temperature and water availability as well as nutritional conditions such as phytohormones, carbohydrates and nitrogen (Bernier *et al*. 1993). The involvement of these factors, extensively analyzed by *in vivo* studies, has been found to be also critical for *in vitro* flowering (Taji *et al*. 2002; Taylor and van Staden 2006). Even if the influence of plant growth regulators has been largely investigated, the results are not always concordant. Cytokinins, mainly BA, are widely referred to as the most successful plant hormone for *in vitro* flowering induction in various species, namely *Streptocarpus nobilis* (Simmonds 1982), *Murraya paniculata* (Jumin and Ahmad 1999), *Dendrocalamus giganteus* (Ramanayake *et al*. 2001), bitter melon (Wang *et al*. 2001), *Panax ginseng* (Lin *et al*. 2005) and *Kniphofia leucocephala* (Taylor *et al*. 2005). However, on the contrary, some reports point to an inhibitory effect of cytokinins on flowering (Dickens and van Staden 1990; Koh and Loh 2000). Otherwise, auxins are generally inhibitory (Simmonds 1982 and citations therein; Kostenyuk *et al*. 1999; Galoch *et al*. 2002; Lin *et al*. 2005).

Our results showed that the combined action of a cytokinin (BA) and an auxin (NAA or IBA) promoted *in vitro* flowering of shoots derived from twin-scale explants. Similar results were described for *Chamomilla recutita* (Kintzios and Michaelakis 1999); in fact, plantlets regenerated from embryos through somatic embryogenesis flowered *in vitro* on a medium supplemented with 8.87 μM BA and 1.07 μM NAA. As in *N. triandrus*, the concentration of BA was higher than that of auxin. Moreover, the interaction of auxin and cytokinin for *in vitro* flowering had been demonstrated for *Bambusa edulis* (Lin *et al*. 2003), although high auxin/cytokinin ratio reduced *in vitro* reproductive growth. For six rose cultivars (Wang GY *et al*. 2002), the most efficient flower bud initiation was achieved on media supplemented with auxin and cytokinin (0.54 μM NAA and 2.27 μM thidiazuron, or 0.54 μM NAA and 2.28 μM zeatin). As was also emphasized for *Dendrobium sonia* and for other orchid species (Sudhakaran *et al.* 2006 and citations therein) cytokinins stimulated *in vitro* flowering but the addition of an auxin improved the induction rate. In some species, the inhibitory action of auxins was negated by cytokinin application, which points to the regulatory role of auxins and cytokinins in flowering (Taji *et al*. 2002).

In addition, recent studies (Taylor and van Staden 2006) stated that *in vitro* flower production largely depends on the timing as well as on the optimal concentration of the applied growth regulators. The development of normal flowers that we observed seems to recommend the removal of cytokinin from the medium after flower bud induction and the maintenance of auxin for flowers' development. As stated by Dickens and van Staden (1990), cytokinins play an important role in the induction of flowering and auxins are essential to support the growth of flowers. It should be also emphasized that in this cytokinin-free medium, the concentration of sucrose was increased from 3% to 9%. This factor may also be important in the development of normal flowers *in vitro*, since high concentrations of carbohydrates in culture media had previously considerably improved flower induction in *Pharbitis* (Ishioka *et al*. 1991) and buckwheat (Kachonpadungkitii *et al*. 2001).

An important factor regulating *in vitro* flowering in *N. triandurs* was temperature. Sub-culturing on medium with NAA and 9% sucrose promoted normal flowering only at 18ºC, while the development at 25ºC on the same medium led to the flowers' abortion. Similarly, previous reports describe the stimulation of flowering by a moderately cold temperature: in *Citrus limon*, vegetative buds flowered *in vitro* when cultured at 14 to 20ºC but they did not flower at 25ºC (Tisserat *et al*. 1990) and in long-term subcultured pear shoots flowering occurred at 15ºC but not at 26ºC (Harada and Murai 1998). It was also stated that the temperature under which twin-scale explants of *N. triandrus* were cultured, as well as the level of auxins and cytokinins, conditioned the induction and proliferation of shoots (Santos I *et al*. 1998). In addition, *Narcissus* development *in vivo* occurs at a similar temperature, at 18ºC (Hanks 1993).

In our material the most striking difference between abnormal flowers developed at 25ºC and normal ones at 18ºC was the behaviour of tapetal cells as well as the architecture of the pollen grain wall. In contrast to abnormal flowers developed at 25ºC, those produced at 18ºC displayed a well-organized tapetal layer during the initial phases of microsporogenesis and the pollen grain wall had the same pattern as that of *in vivo* grown plants. Results concerning the morphology of *in vitro* developed anthers are scarce. *In vitro* flowers morphologically comparable to the *in vivo* ones, although smaller in size, had been found in *Bambusa arundinacea* (Nadgauda *et al*. 1997) and *Gentiana triflora* (Zhang and Leung 2000). In the latter species, pollen grains from both *in vivo* and *in vitro* flowers appeared to be very similar, as revealed by scanning electron microscopy, although the *in vitro* pollen wall of the above referred to bamboo species (Nadgauda *et al*. 1997) showed some discrepancies in comparison to the *in vivo* pollen. The development of pollen requires the expression of genes in both gametophytic and sporophytic tissues (McCormick 1993). Gene products localized in the tapetum are expressed only during specific stages of the formation of outer pollen wall and there is evidence of a biochemical and genetic link between tapetum function and pollen wall ontogeny (Foster *et al*. 2001; Wang A. *et al*. 2002). The importance of the tapetum in pollen development has been demonstrated by genetic approaches. Mutations of tapetum specific genes resulted in its aberrant development and precocious degeneration followed by extensive pollen abortion (Koltunow *et al*. 1990; Wilson *et al*. 2001; Kapoor *et al*. 2002). In flowers of *N. triandrus* developed under 25ºC, the observed precocious degeneration of the tapetal layer was probably responsible for the malformation of the exinic wall and for pollen abortion.

In conclusion, the results obtained in the present work indicate that temperature is a critical factor for the *in vitro* production of normal *Narcissus* flowers. The importance of medium composition, namely, the balance of growth regulators and the carbohydrate concentration, in the transition of the vegetative growth to the reproductive stage, should also be taken into consideration. The data which we report is, to our knowledge, the first microscopic study of anthers and pollen developed during *in vitro* flowering of *Narcissus*, are also of utmost importance for the *in vitro* production of bulbous plants' flowers, widely used for commercial purposes.

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