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 eradicating 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.
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 Na₂Na 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 microscope. 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 et al. 1998), we studied the ontogenic process of both anthers and pollen during in vitro flowerng. 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 whors of radial tepals sharply deflected and two whors 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-
kiin 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). Although the influence of the medium composition, namely, the balance of 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 in various species, namely Streptocarpus nobilis (Simmonds 1982), Murraya paniculata (Jumin and Ahmad 1999), Dendrobulum giganteum (Ramanayake et al. 2001), bitter melon (Wang et al. 2001), Panax ginseng (Lin et al. 2005) and Kniphofia leucocphala (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 roots derived of several species. 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 (Taylor et al. 2005), although high auxin/ cytokinin ratio reduced in vitro reproductive growth. For six rose cultivars (Wang 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 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 additional 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 and the applied growth regulators. The development of normal flowers that were 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%, which may 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 (Kachonpadungkitti et al. 2001).

An important factor regulating in vitro flowering in N. triandrus was temperature. Sub-culturing on medium with NAA and 9% sucrose promoted normal flowering only at 18°C, while their development at 25°C 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 20°C but they did not flower at 25°C (Tisserat et al. 1990) and in long-term subcultured pear shoots flowering occurred at 15°C but not at 26°C (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 vitro occurs at a similar temperature, at 18°C (Hanks 1993).

In our material the most striking difference between abnormal flowers developed at 25°C and normal ones at 18°C was the behaviour of tapetal cells as well as the architecture of the pollen grain wall. In contrast to abnormal flowers developed at 25°C, those produced at 18°C displayed a well-developed tapetal layer during the initial phases of microsporogenesis and the pollen grain wall had the same pattern as that of in vitro 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 the formation of in vitro flowers' pollen 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 25°C, 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.

REFERENCES


Another and pollen development during in vitro flowering. Santos et al.


