Development of adventitious shoots from *in vitro* grown *Cydonia oblonga* leaves as influenced by different cytokinins and treatment duration

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Abstract

The effects of three cytokinins, kinetin 4.5 μM (Kin), 6-benzylaminopurine 4.5 μM (BA) and N-phenyl-N'-1,2,3-thiadiazol-5-yl-urea 4.5 μM (TDZ), and the effects of different treatment duration on the regeneration of adventitious shoots from quince (*Cydonia oblonga* Mill.) leaves were studied. In a first experiment, leaves treated with Kin for 0, 8, 16 and 24 d were transferred to BA or TDZ-containing growth medium. In a second experiment TDZ applied for 0, 4, 8, 12, 16 and 24 d was followed by BA. All treatments included 0.5 μM α-naphthaleneacetic acid (NAA). In the sequence Kin-BA, the production of adventitious shoots decreased and reddish-coloured nodular structures (RNS) of meristematic appearance increased with increasing duration of Kin treatment, while somatic embryo formation was optimal at 8 d. In the Kin-TDZ sequence, shoot production was initially pronounced, but it declined with increasing duration of the Kin treatment, while the number of roots, somatic embryos and RNS increased. TDZ-BA treatments induced marked shoot production, which gradually increased with increasing duration of TDZ treatment. The presence of TDZ and a long treatment duration appeared to be very important factors in inducing caulogenesis. Kin appeared to be effective in shoot induction but not in shoot development; the results of this work demonstrate that RNS were adventitious shoots blocked at an early developmental stage on account of insufficient cytokinin activity. BA was less effective than TDZ in inducing shoot regeneration. Finally, both Kin and BA applied after 2,4-D treatment promoted somatic embryo induction.

Additional key words: adventitious roots; cytokinin activity, *in vitro* morphogenesis, somatic embryos, tissue culture.

Introduction

Cytokinins can induce different cellular responses, and their effects may differ according to both genotype sensitivity to a specific cytokinin and cytokinin activity, the latter depending on the cytokinin potential in stimulating cell division in axillary or adventitious meristems. It is known that cytokinins, such as kinetin, 6-benzylaminopurine (BA) and N-phenyl-N'-1,2,3-thidiazol-5-yl-urea (thidiazuron, TDZ), can differ appreciably in the regeneration response they induce (Fiola *et al*. 1990, Huetteman and Preece 1993, Khanam *et al*. 2000). Thus, since cytokinin is very often applied in combination with auxin, the final response can be expected to differ according to the cytokinin/auxin ratio and to the biological activity of the cytokinin utilised as well.

The leaves of the quince (*Cydonia oblonga* Mill.) clone BA 29 grown *in vitro* had the capacity to simultaneously regenerate somatic embryos and adventitious roots after a 2-d treatment with 2,4-dichlorophenoxyacetic acid (2,4-D) followed by incubation on a kinetin and NAA-containing medium (D'Onofrio *et al.* 1998, D'Onofrio and Morini 2003/4). Some factors, for instance, growth regulator combination and treatment duration (unpublished data), and salt stress induced by NaCl (D'Onofrio and Morini 2002), modified the frequency of such morphogenic structures. Together with somatic embryos and roots, reddish-coloured nodular structures (RNS) were observed that were probably undeveloped adventitious shoots (Morini *et al*. 2000).

Received 24 June 2003, *accepted* 27 May 2004.
Abbreviations: BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid; RNS - reddish nodular structures; TDZ - thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-yl-urea).
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The present research investigated the effects of different cytokinin combinations, applied for different durations, on induction and development of adventitious shoots from quince leaves grown *in vitro*.

Materials and methods

Young leaves, roughly 10 mm in length, of *Cydonia oblonga* Mill. clone BA 29 were collected from the three apical nodes of proliferating shoots established under *in vitro* conditions for about three years. They were incubated for 2 d in liquid MS (Murashige and Skoog 1962) medium containing 1 mg dm^{-3} thiamine-HCl, 100 mg dm^{-3} myo-inositol, 33.64 mg dm^{-3} $FeSO_4$, 44.67 mg dm^{-3} Na_2EDTA, 3 % (m/v) sucrose and 11.3 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The pH was adjusted to 5.5 prior to autoclaving. Incubation was performed in Erlenmeyer flasks placed on a shaker at 70 rpm, under white light (irradiance of 45 ± 5 μmol m^{-2} s^{-1}, photoperiod 16 h). The leaves were then transplanted to fresh medium of the same composition, solidified with 2.5 g dm^{-3} of *Gelrite* (*Sigma*, St. Louis, USA), without 2,4-D, but in the presence of different cytokinin combinations and for different treatment duration as reported in the following experiments.

Experiment 1: Leaves were incubated on 4.5 μM kinetin (Kin) and 0.5 μM α-naphthalenacetic acid (NAA) (Kin + NAA) for 0, 8, 16 or 24 d and subsequently transferred to culture medium containing *A*) 4.5 μM 6-benzylaminopurine (BA) and 0.5 μM NAA (BA + NAA) or *B*) 4.5 μM thidiazuron (TDZ) and 0.5 μM NAA (TDZ + NAA).

Experiment 2: Leaves were incubated on 4.5 μM TDZ and 0.5 μM NAA (TDZ + NAA) for 0, 4, 8, 16, 20 or 24 d, and subsequently transferred to culture medium containing 4.5 μM BA and 0.5 μM NAA (BA + NAA).

After 25 d of culture, the leaves of all the above-mentioned treatments and experiments were transferred to fresh culture medium and incubated for another 25 d. The growth chamber temperature was 24 ± 1 °C, irradiance 45 ± 5 μmol m^{-2} s^{-1} and 16-h photoperiod. Each treatment in each of the three experiments was applied to 50 leaves, arranged in groups of 10 per Petri dishes (6 cm diameter), with adaxial surfaces in contact with the growth medium. At the end of the incubation period, percentage of caulogenic, rhizogenic, and embryogenic leaves and number of shoots, roots and somatic embryos per explant were recorded. Reddish-coloured nodular structures (RNS) were also observed, often in compact groups that rendered the counting very difficult. Thus RNS were evaluated by using the following ranks representing the percentage of abaxial leaf surface covered by such structures: rank 1 for 0 - 20 %, rank 2 for 21 - 40 %, rank 3 for 41 - 60 %, rank 4 for 61 - 80 % and rank 5 for 81 - 100 %.

Percentage values, after angular transformation, and number of different morphogenic structures were submitted to analysis of variance. Two-by-two differences were tested by applying Duncan's multiple range test. RNS production was analysed by the approximated nonparametric Kruskal-Wallis test (Helsel and Hirsch 1995). Two-by-two comparisons were performed by applying a nonparametric LSD test on average ranks.

Results

The proportions found between roots, somatic embryos and shoots, regenerated often by the same explant, were modified by cytokinin combinations and treatment duration.

Direct transfer from 2,4-D to BA + NAA culture medium, without any intermediate treatment with Kin + NAA, produced the highest percentage of caulogenic leaves and the highest number of adventitious shoots per explant (Fig. 1*A*,*B*). On the contrary, adventitious shoot regeneration appeared to be negatively influenced by an intermediate 8-d incubation treatment in the presence of Kin + NAA. With the 16-d treatment of this latter cytokinin combination, no shoot was detected. The percentage of RNS-producing leaves and the number of RNS per explant (Fig. 1*A*,*B*) increased with increasing duration of Kin + NAA treatment. Rhizogenic and embryogenic leaves were observed at all transfer times but the highest percentages were obtained on the 8th d of treatment with Kin + NAA, while the number of roots and somatic embryos per responding explant was higher with 0 d of Kin + NAA treatment and decreased with increasing treatment duration (Fig. 1*A*,*B*).

Leaves transferred directly from 2,4-D to medium containing TDZ + NAA, without intermediate treatments with Kin + NAA, regenerated only adventitious shoots (Fig. 1*C*). The application of an intermediate treatment with Kin + NAA decreased shoot regeneration which was more pronounced with increasing treatment duration. No shoots were regenerated when the leaves were treated with Kin + NAA for 24 d. Meanwhile, root and somatic embryo regenerating leaves were observed from the 8th d of culturing, and increased with increasing duration of Kin + NAA treatment. The number of RNS-producing leaves also increased with prolonging Kin + NAA

treatment and after 24 d of culturing on this cytokinin combination, most leaves produced these structures (Fig. 1*C*). Similar results were observed for the number of roots, shoots, and somatic embryos per leaf (Fig. 1*D*).

Leaves treated with 2,4-D and incubated for different periods on TDZ + NAA regenerated mainly shoots (Fig. 2*A*). This effect increased dramatically with increasing the duration of TDZ + NAA treatment. Somatic embryos, roots and RNS were observed when the leaves were directly transferred from 2,4-D to BA + NAA, without intermediate treatments with TDZ + NAA. At the 4th d of treatment with TDZ + NAA, the number of leaves producing somatic embryos, roots and RNS decreased and disappeared by the 8th - 12th d, while the number of caulogenic leaves increased markedly, reaching 100 % on the 24th day of culturing on TDZ + NAA (Fig. 2*A*). A similar trend was observed for the number of roots, somatic embryos, shoots and RNS per leaf (Fig. 2*B*).

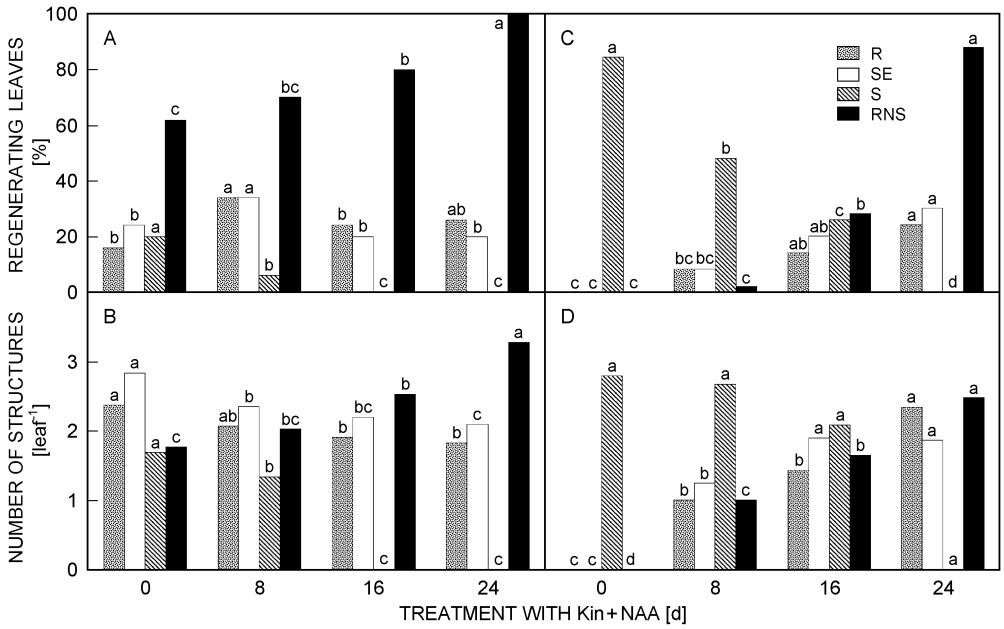

Fig. 1. Regeneration of quince BA 29 leaves treated with Kin 4.5 μM and NAA 0.5 μM for 0, 8, 16 and 24 d and transferred each time to BA 4.5 μM and NAA 0.5 μM (*A*, *B*) or to TDZ 4,5 μM and NAA 0.5 μM (*C*, *D*). *A*, *C* - percentages of leaves regenerating adventitious shoots (S), roots (R), somatic embryos (SE) and reddish nodular structures (RNS). *B*, *D* - mean number of morphogenic structures per regenerating leaf. In each figure, different letters indicate statistically different values ($P < 0.05$) according to Duncan's test within each type of regenerating leaf (*A*, *C*) or within each type of morphogenic structure (*B*, *D*).

Discussion

The results obtained in these experiments indicate that consecutive treatments of variable duration with Kin and BA, Kin and TDZ or TDZ and BA lead to pronounced differences in regeneration response in the quince leaf. The literature contains reports suggesting that these three cytokinins can be ranked on a scale of increasing biological activity, from Kin to BA to TDZ (Fiola *et al*. 1990, Khanam *et al.* 2000, Chengalrayan and Gallo-Meagher 2001). Thus, their effect was to be expected to vary according to the cytokinin requirement of a given regeneration process.

In our work, the application of TDZ, a compound with high cytokinin-like activity, determined a dramatic increase in the production of adventitious shoots with increasing treatment duration. This demonstrates that the shoot regeneration process needed the presence of a cytokinin of high biological activity and that the cytokinin treatment had to be applied for a period as long as about 20 - 25 d to reach the maximum response. In our experiments, the treatment with TDZ followed by BA had induced already on the 4th d of culturing, a pronounced production of adventitious shoots which were the main morphogenic structures regenerated at this time (Fig. 2). These results are in agreement with those obtained by Biondi and Thorpe (1982), who observed that meristematic tissue formation and subsequent shoot differentiation required the presence of cytokinin in the first 3 d of culturing, the optimum being reached with 21 d of treatment.

The high cytokinin requirement of the caulogenic process in quince leaves is also confirmed by the variations in the amount of RNS obtained with different

duration of treatment with Kin or TDZ. In the sequences Kin-BA, RNS production was very high and continued to increase when Kin was applied with increasing duration. In the sequence Kin-TDZ, the amount of RNS was still increasing with corresponding increasing treatment

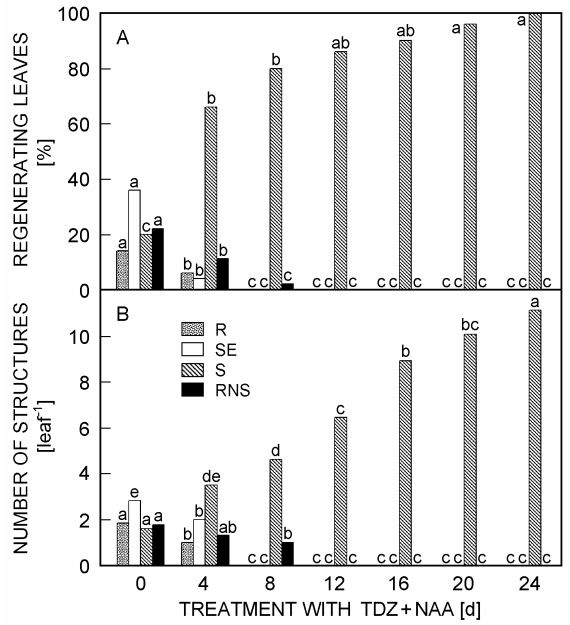

Fig. 2. *A* - Percentages of quince BA 29 leaves regenerating adventitious shoots (S), roots (R), somatic embryos (SE) and producing reddish nodular structures (RNS), after 2 d treatment with 2,4-D and 0, 4, 8, 16, 20 and 24 days of treatment with TDZ 4.5 μM and NAA 0.5 μM; at each time the leaves were then transferred to a growth medium containing a combination of BA 4.5 μM and NAA 0.5 μM. Percentage values were calculated without taking into account whether a single leaf produced different morphogenic structures. *B* - Mean number of morphogenic structures per regenerating leaf. In both figures, different letters indicate statistically different values ($P < 0.05$) according to Duncan's test within each type of regenerating leaf (*A*) or within each type of morphogenic structure (*B*).

applications but the values were lower than in the presence of BA, demonstrating that TDZ exerted a negative effect on RNS production. In the sequence TDZ-BA, RNS were, in general, present in the absence of TDZ in the culture medium, but they decreased just after a brief treatment (4 d) with this compound (Fig. 2). At the 8th day of culturing, leaves with RNS were still decreasing and RNS disappeared thereafter. In all the experiments described above, RNS production decreased while adventitious shoot production increased. Such results suggest a close relationship between RNS and adventitious shoot differentiation, and indicate that the RNS observed in a number of other experiments (Morini *et al.* 2000), were caulogenic structures that were aborted at an early stage of development on account of low activity of the cytokinin applied. This interpretation is also corroborated by the observation that prolonged treatments with Kin, even if the leaves were successively incubated in the presence of BA or TDZ, induced a progressive increase in RNS production, while shoot production decreased drastically (Fig. 1). This also indicates that a cytokinin with high biological activity is required from the beginning of the regeneration process to allow an undisturbed shoot differentiation. Finally, BA was shown to have less effect on shoot induction and differentiation compared to TDZ, as the results obtained with both cytokinins applied without intermediate Kin treatment (0 d treatment in Fig. 1*A*,*C*) demonstrate.

Regarding cytokinin combination effects on somatic embryo regeneration, the results obtained in the present experiments suggest that the latter is initially favoured by the presence of a cytokinin with relatively low or moderate biological activity in the auxin/cytokinin ratio, *i.e*. by conditions that neither strongly inhibit rhizogenesis nor particularly stimulate caulogenesis. Evidence for this hypothesis is found in *1*) the presence of somatic embryos in all Kin + NAA treatments in the sequence Kin - BA (Fig. 1*A*,*B*), *2*) the appearance and the increasing production of somatic embryos from the 8th d of culturing on Kin + NAA in the sequence Kin - TDZ (Fig. 1*C*,*D*), and *3*) the presence of somatic embryos on leaves directly transferred from 2,4-D to BA + NAA medium or when TDZ was applied for 4 d only, in the sequence TDZ - BA (Fig. 2*A*,*B*). In our experiments TDZ exerted significant negative effects on somatic embryo regeneration. This was in contrast with the results obtained on *Fraxinus americana* (Bates *et al.* 1992), *Cayratia japonica* (Zhou *et al.* 1994), *Arachis hypogaea* (Murthy *et al.* 1995), and *Beta vulgaris* (Zhang *et al.* 2001). Most somatic embryos observed in our experiments showed an abnormal morphological appearance (cotyledon fasciation) regardless of the treatment applied, while adventitious roots and shoots appeared to be normally shaped. Only a few somatic embryos presented the typical embryonal bipolarity and more regular morphology.

Conclusion: These results indicate that the presence of a cytokinin with high biological activity, as TDZ, is required to promote induction and differentiation of adventitious shoots in young quince leaves collected from *in vitro*-grown shoots. Treatment duration must be fairly prolonged to allow the caulogenic process to evolve and lead to the maximum production of morphologically normal adventitious shoots. TDZ has been shown to be more effective than BA in inducing adventitious shoot formation, as also observed in other species such as *Rubus* (Fiola *et al.* 1990). From a practical point of view, the importance of this finding is that by choosing the right cytokinin and treatment duration, the formation of adventitious shoots in quince leaves (Fig. 2) can be substantially increased. These shoots elongated rapidly with no difficulty and could be micropropagated and rooted (unpublished data). With respect to somatic

embryo formation, a cytokinin with relatively low or intermediate biological activity applied after 2,4-D treatment appeared to perform more efficiently in the embryogenic induction process.

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