BIOLOGIA PLANTARUM 47 (3): 321-325, 2003/4

# Simultaneous regeneration of different morphogenic structures from quince leaves as affected by growth regulator combination and treatment length

C. D'ONOFRIO and S. MORINI*

*Dipartimento di Coltivazione e Difesa delle Specie Legnose "G. Scaramuzzi", Sezione Coltivazioni Arboree, Università di Pisa, Via del Borghetto 80, I-56124 Pisa, Italy*

## Abstract

Experiments were performed to evaluate the capacity of quince (*Cydonia oblonga* Mill.) leaves to regenerate somatic embryos and shoots and/or roots simultaneously. Leaves, treated for 2 d in liquid medium containing 2.5 mg $dm^{-3}$ 2,4-dichlorophenoxyacetic acid were cultured for 0, 3, 6, 9, 12, 15, 18, 21 d on a gelled medium supplemented with 1 mg $dm^{-3}$ kinetin (Kin) and 0.1 mg $dm^{-3}$ naphthalenacetic acid (NAA) and were transferred to a medium either without growth regulator (GR-) or containing 0.6 mg $dm^{-3}$ 6-benzylaminopurine (BA) + 0.2 mg $dm^{-3}$ gibberellic acid ($GA_3$) + 0.06 mg $dm^{-3}$ indole-3-butyric acid (IBA) (GR+). Leaves producing somatic embryos (SEs) only, or adventitious roots (Rs) only, or SEs+Rs simultaneously, were detected on GR- culture medium; on GR+ medium, leaves producing adventitious shoots (Ss) only, SEs+Ss or SEs+Rs+Ss simultaneously, also appeared. Leaves producing both Ss+Rs were never detected. Proportions among the various types of regenerating leaves varied according to both the length of Kin+NAA treatment and the presence or absence of GR in the transfer medium. The greatest variations, both on GR- and on GR+, took place within the first 9 d of culturing on Kin+NAA. After this period, no further substantial differences in the trend of each type of regenerating leaf were observed. The length of the treatment with Kin+NAA also modified the proportions between the different types of morphogenic structures.

*Additional key words*: adventitious shoot, adventitious root, cytokinin, *Cydonia oblonga*, morphogenesis, somatic embryo.

## Introduction

The factors inducing *in vitro* morphogenesis are still not fully known. Following certain stimuli, cells undertake regeneration processes (caulogenesis, rhizogenesis, somatic embryogenesis), either singly or in groups (Reinert *et al.* 1977), but the physiological mechanisms responsible for a specific event and the reason why a certain morphogenic process begins instead of another remains to be clarified.

According to Christianson (1985), the cells of a tissue or an organ may respond to different factors by becoming activated in accordance with a specific developmental system. Cells controlled by different systems should be arranged differently on a three-dimensional response surface (Christianson 1985). Each cell is thought to respond differently to external stimuli, depending on its position on the three-dimensional surface. Thus cells that have different developmental systems activated are likely to respond differently to the same stimulus and consequently undertake different morphogenic processes.

Previous research on the morphogenesis of woody fruit-tree species from family *Rosaceae* carried out in our laboratory highlighted the fact that quince leaves cultured *in vitro* regenerated somatic embryos and roots following the same growth regulator treatment (D'Onofrio *et al.* 1998, Morini *et al.* 2000). These morphogenic structures were produced by different leaves or simultaneously by the same leaf and the proportions among the different leaf groups appeared to be influenced by light quality and

*Received* 13 May 2002, *accepted* 27 March 2003.

*Abbreviations*: BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; $GA_3$ - gibberellic acid; GR+ - medium containing: 0.6 mg $dm^{-3}$ BA, 0.2 mg $dm^{-3}$ $GA_3$ and 0.06 mg $dm^{-3}$ IBA; GR- - free growth regulators medium; IBA - indole-3-butyric acid; Kin - kinetin; NAA - naphthalenacetic acid; R - root; S - shoot; SE - somatic embryo.

*Author for correspondence; fax: (+39) 50-544420, e-mail: smorini@agr.unipi.it

saline stress (D'Onofrio *et al.* 1998, D'Onofrio and Morini 2002a,b). Moreover, replacing kinetin with 6-benzylaminopurine in the growth medium, the simultaneous production of somatic embryos, adventitious shoots and roots was also observed (D'Onofrio and Morini (unpublished).

In this work we studied the effects of different growth regulator combinations and treatment lengths, on the capacity of quince leaves to simultaneously regenerate different morphogenic structures.

## Materials and methods

Leaves of quince (*Cydonia oblonga* Mill.), clone BA 29, were used as explants. They were collected from the three apical nodes of proliferating shoots cultured on DKW (Driver and Kuniyuki 1984) medium supplemented with 1 mg $dm^{-3}$ thiamine-HCl, 100 mg $dm^{-3}$ myo-inositol, 33.64 mg $dm^{-3}$ $FeSO_4$, 44.67 mg $dm^{-3}$ $Na_2EDTA$, 30 g $dm^{-3}$ sucrose, 1.5 mg $dm^{-3}$ 6-benzylaminopurine (BA), and 1.5 mg $dm^{-3}$ of BA-riboside. After adjusting pH to 5.6, the medium was supplemented with 4 g $dm^{-3}$ agar and 5 g $dm^{-3}$ *Frimulsion UF Carrageneen* (*Cesalpinia Food*, Bergamo, Italy), followed by sterilisation in autoclave at 120 °C and 101 kPa for 20 min. Shoot cultures were grown in a growth chamber at a temperature of 24 ± 1 °C, under white light (irradiance of 50 ± 5 μmol $m^{-2}$ $s^{-1}$) with a 16-h photoperiod. The cultures were transferred to fresh medium every three weeks.

At sampling, leaves were scored transversally three times on the abaxial leaf surface and shakered at 70 rpm in groups of 50, in liquid induction medium for 2 d with the same temperature and light conditions as adopted for the mother shoot cultures. Murashige and Skoog (1962, MS) medium supplemented with 100 mg $dm^{-3}$ myo-inositol, 0.4 mg $dm^{-3}$ thiamine-HCl, 30 g $dm^{-3}$ of sucrose, 2.5 mg $dm^{-3}$ 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg $dm^{-3}$ $AgNO_3$ as ethylene synthesis inhibitor (Chi and Pua 1989, Roustan *et al.* 1990) was used as induction medium. The pH was adjusted to 5.6 with KOH before sterilisation in autoclave. After 2,4-D treatment, the leaves were transferred in groups of 10- to 6-cm Petri dishes containing 10 $cm^3$ of culture medium gelled with 2.5 g $dm^{-3}$ of *Gelrite* (*Sigma*, St. Louis, USA). In this medium, 2,4-D was replaced with 1 mg $dm^{-3}$ kinetin (Kin) + 0.1 mg $dm^{-3}$ NAA. Leaves were positioned with the adaxial surface in contact with the medium and incubated under red radiation of 20 ± 2 μmol $m^{-2}$ $s^{-1}$ (D'Onofrio *et al.* 1998), 24 ± 1 °C. After 0, 3, 6, 9, 12, 15, 18 and 21 d of Kin+NAA treatment, two groups of 50 leaves (5 replicates of 10 leaves each) were collected: one group was transferred to growth regulator-free MS medium (GR-), and the other to MS medium (GR+) containing 0.6 mg $dm^{-3}$ BA + 0.2 mg $dm^{-3}$ gibberellic acid ($GA_3$) + 0.06 mg $dm^{-3}$ indole-3-butyric acid (IBA). After 25 d of red radiation treatment the cultures were transferred to fresh medium and placed for another 25 d under white light. At the end of this period, the proportions among the leaves regenerating somatic embryos (SE), roots (R) and shoots (S), alone or simultaneously, were calculated for each Kin+NAA treatment length and SEs, Rs and Ss on each leaf were counted. Percentages of leaves, after angular transformation, and number of morphogenic structures were analysed statistically by *ANOVA*. Means were compared by the Duncan's multiple range test.

## Results and discussion

Leaves, even if belonging to the same treatment, regenerated SEs, Rs and Ss, either alone or in combination, as already observed on leaves of the same quince clone (D'Onofrio *et al.* 1998) and *Leucosceptrum canum* (Pal *et al.* 1985). This capacity was modified by the different length of treatment with Kin+NAA and by the presence or absence of GRs in the transfer medium. The physiological mechanisms involved in such behaviour are unknown but the different degree of leaf cell differentiation and a different endogenous hormonal status ot the tissue (Dale 1988, Sinha 1999) possibly had a role. Thus, young leaflets utilized in our experiments could be compared to the three dimensional response surface indicated by Christianson (1985), where cells at different differentiation stages are controlled by different developmental systems which are thought to differ with leaf age. Leaves that simultaneously regenerated different morphogenic structures were probably those characterized by a more heterogeneous differentiation stage, such as the still immature leaves, as compared to leaves that produced a single type of morphogenic structure.

Without Kin+NAA treatment (0 d in Fig. 1*A*) and GR- transfer medium, the leaves subjected only to 2,4-D treatment could be divided into three groups: one, representing roughly 65 % of total morphogenic leaves, regenerating Rs only, and the other two groups, each accounting for about 15 - 20 % of leaves, producing SEs only or SEs+Rs. The Kin+NAA treatments caused substantial modifications in the proportions among the groups of regenerating leaves with increasing treatment length (Fig. 1*A*). The proportion of rhizogenic leaves

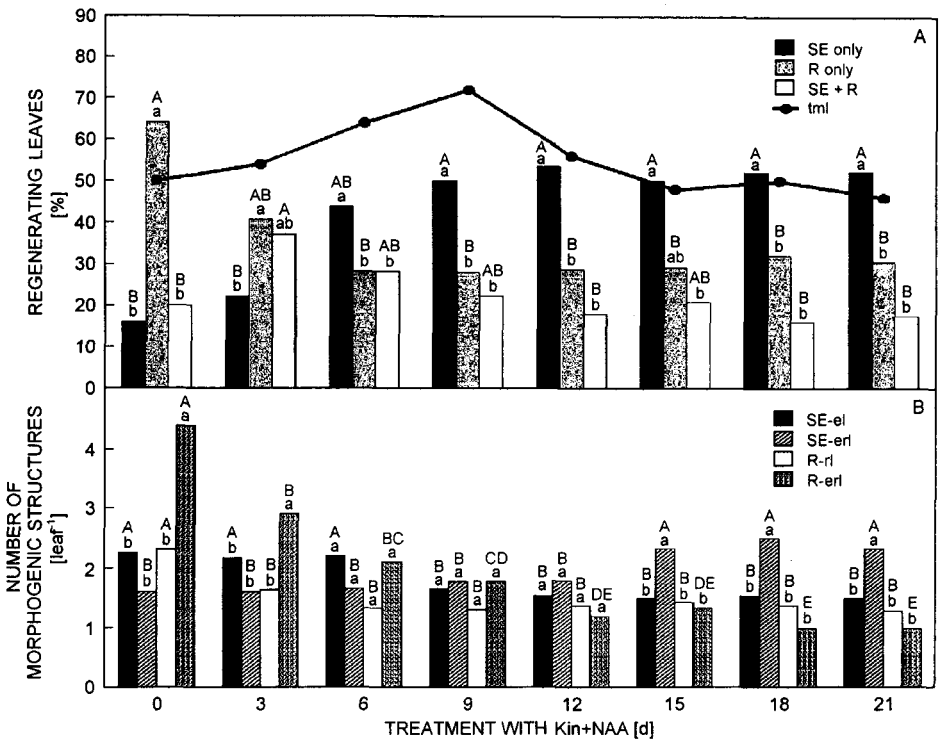


Fig. 1. Proportions among groups of quince leaves regenerating different morphogenic structures (*A*) and number of morphogenic structures per leaf (*B*) recorded after Kin+NAA treatments of different length with subsequent transfer to a growth regulator-free medium (GR-). The proportions are expressed as percentages of the total number of regenerating leaves recorded in each Kin+NAA treatment. Different lower case letters among regenerating leaf types within each Kin+NAA treatment or different capital letters among Kin+NAA treatments within each morphogenic structure type, indicate values statistically different at $P \leq 0.05$. In *A*: SE - somatic embryos; R - roots; SE+R - somatic embryos + roots; tml - total morphogenic leaves. In *B*: SE-el - number of somatic embryos per embryogenic leaf; SE-erl - number of somatic embryos per embryo-rhizogenic leaf; R-rl - number of roots per rhizogenic leaf; R-erl - number of roots per embryo-rhizogenic leaf.

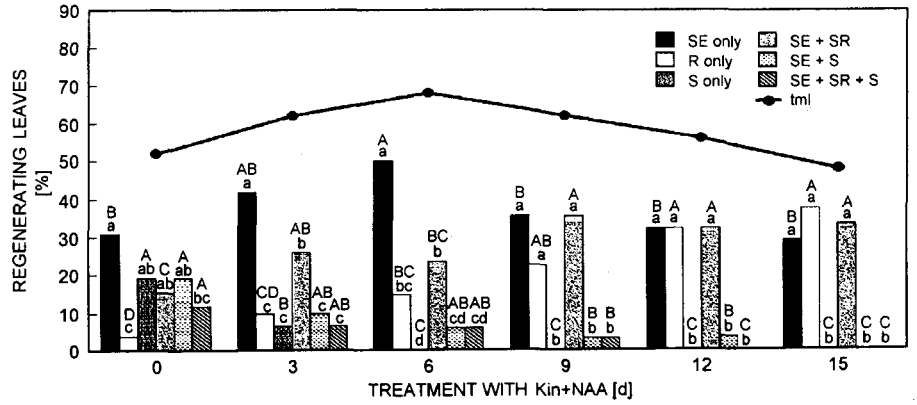


Fig. 2. Proportions among groups of quince leaves regenerating different morphogenic structures recorded after Kin+NAA treatments of different length and subsequent transfer to a growth regulator-enriched (0.6 mg dm$^{-3}$ BA + 0.2 mg dm$^{-3}$ $GA_3$ + 0.06 mg dm$^{-3}$ IBA) medium (GR+). The values are expressed as percentages of the total number of regenerating leaves recorded in each Kin+NAA treatment. Different lower case letters among regenerating leaf types within each Kin+NAA treatment or different capital letters among Kin+NAA treatments within each morphogenic structure type, indicate values statistically different at $P \leq 0.05$. The 18 and 21 d treatments data have been omitted from the figure since they were very similar to those of the 15 d treatment. SE - somatic embryos; R - roots; S - shoots; SE+R - somatic embryos + roots; SE+S - somatic embryos + shoots; SE+S+R - somatic embryos + shoots + roots; tml - total morphogenic leaves.

decreased until the 6th day of treatment and then stabilized at about 30 %, the proportion of embryogenic leaves increased up to 50 % on the 9th day of treatment, and showed no further appreciable variations. SEs+Rs regenerating leaves increased up to the 3rd day of treatment, subsequently decreased to the 12th day and stabilized around 15 - 20 %.

Increasing the length of culturing period on Kin+NAA, the number of SEs on embryogenic leaves decreased while on embryo-rhizogenic leaves they increased. In contrast, Rs diminished on rhizogenic and embryo-rhizogenic leaves (Fig. 1*B*).

The presence of GRs in the transfer medium further amplified explant regeneration capacity: adventitious Ss (Fig. 2) were also observed alone or in combination with SEs or with SEs+Rs; Ss were never observed in combination with Rs. Thus, direct transfer of leaves from 2,4-D treatment to GR+ medium, without intermediate Kin+NAA treatment (0 d in Fig. 2), resulted in the appearance of six groups of regenerating leaves; SE only regenerating leaves were the most represented, in contrast to the GR-experiments where rhizogenic leaves prevailed. Another three leaf groups were each characterised by the production of Ss, or SEs+Rs, or SEs+Ss and accounted for lower but similar percentage values. An even smaller leaf group produced SEs+Rs+Ss simultaneously, and finally the smallest group of leaves, produced Rs only.

The application of Kin+NAA treatments modified the proportions between the various types of regenerating leaves as early as 3 d after the beginning of treatment (Fig. 2). Differences in proportions increased with increasing Kin+NAA treatment length. Thus the proportion of leaves regenerating SEs only, increased until the 6th day of culturing, decreased thereafter and then stabilized at around 30 - 35 % after the 9th day. Leaves regenerating Rs only or SEs+Rs also increased up to the 9th - 12th day and stabilized at around 30 - 35 %. In contrast, leaves that regenerated SEs+Ss or SEs+Ss+Rs decreased up to the 9th - 12th day of culturing on Kin+NAA and then disappeared completely. The proportion of leaves regenerating Ss only, decreased drastically after the 3rd day and totally disappeared by the 6th day of culturing on Kin+NAA.

The number of morphogenic structures regenerated per leaf, in the presence of GRs, showed some variations with varying length of Kin+NAA treatment (Fig. 3). SEs (Fig. 4) tended always to decrease but on embryo-caulogenic leaves they strongly increased up to the 9th day of culturing on Kin+NAA, and subsequently fell to zero on the 15th day (Fig. 3*C*). Rs tended to increase during the second part of culturing period on rhizogenic (Fig. 3*A*) and particularly on embryo-rhizogenic leaves (Fig. 3*B*) while on embryo-rhizo-caulogenic leaves they always decreased (Fig. 3*D*). Ss always decreased with increasing period of culture on Kin+NAA (Fig. 3*A,C,D*).

The most noteworthy variations in proportions between regenerating leaves appeared within the 9th day of culturing on Kin+NAA in both GR- and GR+ experiments. This result demonstrates that this period is the most effective for a given growth regulator combination in modifying leaf regeneration capacity by canalising cells towards one type of regeneration rather

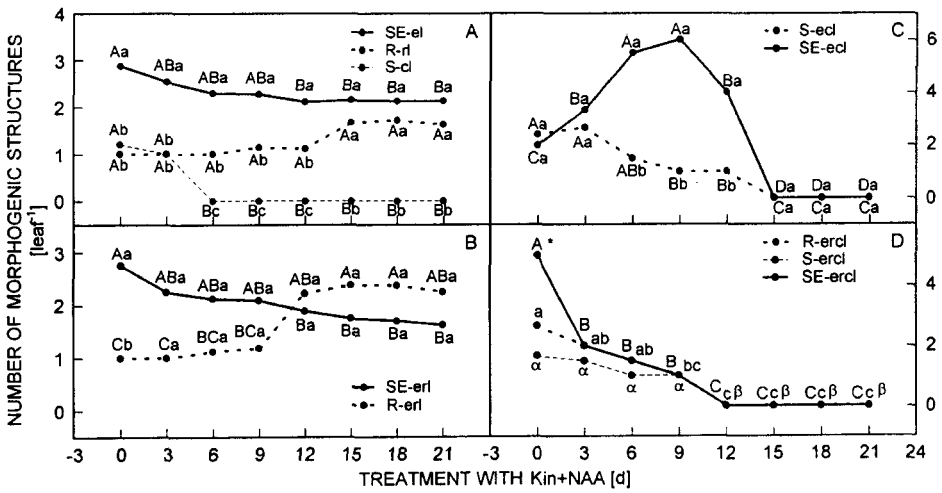


Fig. 3. Number of somatic embryos, roots or shoots per leaf recorded on different types of regenerating leaves, after treatments with Kin+NAA of different length and subsequent transfer to a growth regulator-enriched medium (GR+). In *A*, *B* and *C* lower case or capital letters indicate values statistically different ($P \leq 0.05$) among the number of regenerated structures within each Kin+NAA treatment or among Kin+NAA treatments within each morphogenic structure type, respectively. In *D*, different capital, lower case or Greek letters within each type of morphogenic structures, indicate values statistically different ($P \leq 0.05$) among Kin+NAA treatments. * - statistically different value among morphogenic structure types. SE - somatic embryos; R - adventitious roots; S - adventitious shoots. In: *A* - embryogenic (el), rhizogenic (rl) and caulogenic (cl) leaves; *B* - embryo-rhizogenic leaves (erl); *C* - embryo-caulogenic leaves (ecl); *D* - embryo-rhizo-caulogenic leaves (ercl).

than another. Leaves that at the beginning of culturing period were induced to regenerate Ss, alone or in combination with SEs and SEs+Rs, appeared to be more easily influenced by the length of the Kin+NAA treatment (Fig. 2).

Besides the probable influence of a different degree of leaf cell differentiation in determining the results reported above, the biological activity of the cytokinin applied appeared to be a further important factor. The reduction in R regeneration detected with increasing the Kin+NAA treatment length and in the absence of GR in transfer medium (Fig. 1), was presumably determined by the presence of Kin, which being a weak cytokinin (Khanam *et al.* 2000), accumulated its inhibiting effect on root induction progressively but gradually favoured somatic embryo regeneration. On the contrary, the cytokinin requirement for the embryogenic process appeared to be promptly satisfied when the growth medium was supplemented with the BA, present in GR+ (Fig. 2), characterized by a greater biological activity than Kin. Finally, the production of adventitious Ss on leaves transferred directly from 2,4-D treatment to GR+ (containing BA) medium, and the remarkable shoot reduction recorded after a few days of culture on Kin+NAA (Figs. 2 and 3), would appear to indicate the importance of a cytokinin with high biological activity and of the treatment length stimulating the shoot regeneration process.

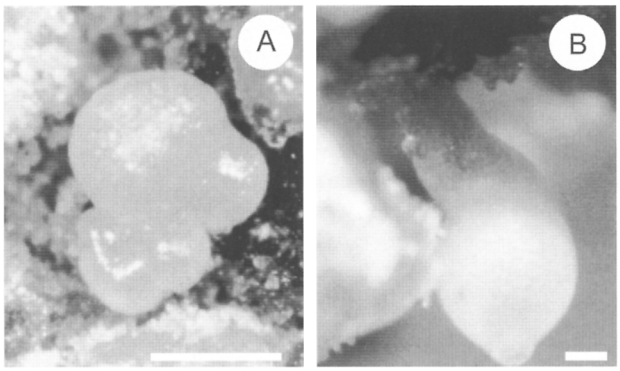


Fig. 4. *A* - somatic embryos at globular stage. *B* - somatic embryo at cotyledonary stage. *Bars* = 500 μm.

The unexpected decreasing number of adventitious Ss, observed with protracting the Kin+NAA treatments when the transferring medium contained BA, occurred simultaneously with a progressive increase of meristematic structures, reddish and with nodular morphology that histological analysis suggested to be similar to precociously aborted shoots (Morini *et al.* 2000). Such structures might be the result of an inadequate biological activity of Kin on the S regeneration process, which possibly caused an abnormal development in some adventitious shoots produced during the first days of culture, rendering them no larger identifiable.

This work highlighted the capacity of quince leaves to regenerate simultaneously different morphogenic structures; this capacity was affected by the length of Kin+NAA treatment, by the presence or absence of growth regulators in the transfer medium and by the biological effectiveness of the cytokinin applied.

## References

Chi, G.L., Pua, E.C.: Ethylene inhibitors enhanced *de novo* shoot regeneration from cotyledons of *Brassica campestris* ssp. *chinensis* (Chinese cabbage) *in vitro*. - Plant Sci. **64**: 243-250, 1989.

Christianson, M.L.: An embryogenic culture of soybean: towards a general theory of somatic embryogenesis. - In: Henke, R.R., Hughes, K.W., Constantin, M.J., Hollaender, A. (ed.): Tissue Culture in Forestry and Agriculture. Pp. 83-103. Plenum Press, New York 1985.

Dale, J.E.: The control of leaf expansion. - Annu. Rev. Plant Physiol. Plant mol. Biol. **39**: 267-295, 1988.

D'Onofrio, C., Morini, S.: Increasing NaCl and $CaCl_2$ concentration in quince leaves growth medium: I. Effects on somatic embryo and root regeneration. - *In Vitro* cell. dev. Biol. Plant **38**: 366-372, 2002a.

D'Onofrio, C., Morini, S.: Increasing NaCl and $CaCl_2$ concentration in quince leaves growth medium: II. Effects on shoot regeneration. - *In Vitro* cell. dev. Biol. Plant **38**: 373-377, 2002b.

D'Onofrio, C., Morini, S., Bellocchi, G.: Effect of light quality on somatic embryogenesis of quince leaves. - Plant Cell Tissue Organ Cult. **53**: 91-98, 1998.

Driver, J.A., Kuniyuki, A.H.: *In vitro* propagation of paradox walnut rootstock. - HortScience **19**: 507-509, 1984.

Khanam, N., Khoo, C., Khan, A.G.: Effects of cytokinin/auxin combination on organogenesis, shoot regeneration and tropane alkaloid production in *Duboisia myoporoides*. - Plant Cell Tissue Organ Cult. **62**: 125-133, 2000.

Morini, S., D'Onofrio, C., Bellocchi, G., Fisichella, M.: Effect of 2,4-D and light quality on callus production and differentiation from *in vitro* cultured quince leaves. - Plant Cell Tissue Organ Cult. **63**: 47-55, 2000.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Pal, A., Banerjee A., Dhar, K.: *In vitro* organogenesis and somatic embryogenesis from leaf explants of *Leucosceptrum canum* Sm. - Plant Cell Rep. **4**: 281-284, 1985.

Reinert, J., Bajaj, Y.P.S., Zbell, B.: Aspects of organization-organogenesis, embryogenesis, cytodifferentiation. - In: Street, H.E. (ed.): Plant Tissue and Cell Culture. Pp. 389-426. Blackwell Scientific Publication, Oxford - London 1977.

Roustan, J.P., Latché, A., Fallot, J.: Control of carrot embryogenesis by $AgNO_3$, an inhibitor of ethylene action: effect on arginine decarboxylase. - Plant Sci. **67**: 89-95, 1990.

Sinha, N.: Leaf development in angiosperms. - Annu. Rev. Plant Physiol. Plant mol. Biol. **50**: 419-446, 1999.