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# Direct embryo formation in leaves of *Camillia japonica* L.

**M. C. Pedroso and M. S. Pais**

Departamento de Biologia Vegetal, Faculdade de Ciências, Universidade de Lisboa, Bloco C2, Piso 1, Campo Grande, 1700 Lisboa, Portugal

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**Summary.** The culture conditions for direct embryo formation in leaves of *Camellia japonica* L. were established. An auxin treatment followed by incubation during 11 days in darkness on diluted Murashige and Skoog modified basal medium induced direct morphogenesis. The number of subcultures, subculture interval and leaf age affected *in vitro* leaf response. The results showed that the cells from a cultured leaf respond differently to the same culture conditions by forming embryos, roots, and non-morphogenic as well as organogenic callus. Direct embryo formation occurred only in the marginal leaf regions. Direct root formation only occurred in a well-defined region of the midrib whereas callus was preferentially formed on the leaf basis. The results suggest the existence of differences in morphogenic competence according to leaf regions. Plantlet regeneration was successfully achieved from somatic embryos and from leaf basis-derived callus, via shoot bud induction.

**Abbreviations:** BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; DTT - dithiothreitol; IAA - indole-3-acetic acid; IBA - indole-3-butyric acid.

## Introduction

*Camellia japonica* L. (*Theaceae*) is the most important ornamental species of the genus *Camellia*. This woody plant, introduced in Europe by Portuguese sailors in 1540, can also be used for oil and wood production. The *in vitro* culture research reported until now is relatively scarce and concerns mainly the development of methods for *in vitro* propagation of this species (for review, Pedroso-Ubach 1991). Somatic embryogenesis was obtained in embryonic tissues (Kato 1986; Vieitez et Barciela 1990), roots (Vieitez *et al.* 1991) and stems of plantlets (Pedroso-Ubach 1991), anthers and microspores (Pedroso-Ubach 1991).

The success of agrobiotechnology depends on controlling plant regeneration. For that reason the study of the molecular basis of morphogenesis, in particular of somatic embryogenesis and rhizogenesis is, at present, one of its major goals. The identification of signals that induce specific responses in certain cells is indispensable for understanding cell competence and determination. Earlier results obtained in our laboratory showed the potential of *C. japonica* to be used as a model system for studying *in vitro* morphogenesis in woody plants. The development of culture systems for a controlled induction of indirect and direct embryogenesis and rhizogenesis was the first step to that approach.

In this paper we report the establishment of culture conditions for direct embryo formation and plant regeneration from leaves of *Camellia japonica*.

## Material and Methods

### *Plant material*

Axenic shoot cultures from juvenile material of an adult plant of *Camellia japonica* cv. Elegans (> 50 years old) were used as a source of leaves. Plant material was harvested in a private garden in S.Paio (Gouveia, Portugal). Shoot cultures were established from axillary buds from this plant as described by Pedroso-Ubach (1991) as well as from plantlets from *in vitro* germinated seeds (immature and ripe) of the same origin. Independent experiments, carried out simultaneously, were performed using leaves isolated from shoots of both origins. A seed-derived clone initiated and established by A. Vieitez and A. Ballester (CSIC, Santiago de Compostela, Spain), described by us as Clone Santiago, was used for comparison in some assays. First (1st), second (2nd) and third (3rd) entire leaves, isolated from shoots subcultured every 12 weeks, were preferentially used for direct root and embryo formation. We considered as first leaf the one below the apical bud.

*Correspondence to:* M. C. Pedroso

*Culture conditions*

Unless otherwise stated, the culture medium used was the basal medium (macro and micronutrients and vitamins) of Murashige and Skoog (1962) modified by replacing ferrous sulphate by ferric citrate and adding DTT both at 5 mg/L (modified MS medium) (Pedroso-Ubach 1991). The pH was adjusted to 5.5 before autoclaving. For culture on solid medium, agar at 7 g/L was added to all media. On solid medium, the leaves were placed with the abaxial surface down. Cultures in liquid medium were shaken at 70-80 rpm. Cultures were maintained at 24±1° C, under light (16 h day photoperiod; 26 $\mu E.m^{-2}.s^{-1}$) provided by cool fluorescent lamps (Grolux Sylvania). Glass culture vessels (300 $cm^3$) with opaque plastic closure (light intensity reduced to 30%) were used for embryo formation and plantlet regeneration.

*Seed germination*

Immature capsules and ripe seeds were surface sterilized by immersion in full strength domestic bleach (5% chlorine plus detergent) during 20 min, rinsed several times with sterile distilled water and dissected in a flow bench. Ripe seeds were opened with a nutcracker. When phenolic compound production was high, embryo isolation was performed in 5 ml of culture medium with 2% (w/v) polyvinylpolypyrrolidone (PVPP). The isolated immature and mature zygotic embryos were immediately cultured on 10 ml of solid culture medium in glass tubes (135 mm x 27 mm) closed with double aluminum foil. The germination medium (MS/2-25) consisted of modified half-strength MS medium with 25 g/L D-glucose; pH 5.5. The cultures were kept in darkness until the onset of germination and then transferred to light. Two weeks later sprouts were transferred to MS/2-25 medium plus BA at 1mg/L. The seedlings obtained were then subcultured to micropropagation medium. This plant material was submitted to at least four multiplication cycles before being used as leaf source.

*Micropropagation*

Shoot cultures were maintained in glass vessels (500 $cm^3$) on solid MS28 medium (micropropagation medium), modified MS medium with 25 g/L D-glucose, 1 mg/L BA, and 0.1 mg/L IBA or IAA; pH 5.7. Shoot cultures were maintained by transferring the cultures to fresh medium every 6 weeks, subculturing nodal segments (1-3 cm) and apical shoot tips every 12 weeks, unless otherwise stated (Fig. 1).

*Induction of direct embryo and root formation*

Entire leaves were immersed in an IBA solution at 1 g/L for 20 min., transferred to solid or liquid MS/2-25 medium and incubated in darkness for 11 days (induction treatment). The cultures were then transferred to light (12 $\mu E.m^{-2}.s^{-1}$). Control experiments were performed as described above, but without the auxin treatment. Six weeks after culture initiation, the cultures were transferred to solid MS28 medium for somatic embryo maturation and germination. Leaf-derived calluses were isolated and transferred to the same medium for shoot regeneration. The assays were repeated 12 times with 70 to 200 leaves per assay. The effect of the number of subcultures and subculture interval on leaf response, expressed as the percentage of leaf response (leaves forming callus, embryos and roots), was recorded 4 weeks after the induction treatment. The effect of the number of subcultures (4, 8, and 10-15) was studied using shoot cultures subcultured regularly every 12 weeks. The effect of subculture interval was studied using shoots cultured on MS28 medium for 12, 16, and 20 weeks since the last subculture without transfer to fresh medium. All the leaves of the shoots (8-11 leaves per shoot) were used in these experiments. Similarly, the effect of leaf age was also studied by comparing leaf response on cultures of $1^{st}$, $2^{nd}$ and $3^{rd}$ leaves and on cultures of $1^{st}$ to the $9^{th}$ or $10^{th}$ shoot leaf (all leaves). We considered "leaf age" the different ages of the leaves within a shoot. Shoots established 2 years before and subcultured every 12 weeks were used in this experiment. These three groups of experiments were performed independently and each repeated 4 to 6 times. The values presented are the mean ± SD.

## Results and Discussion

*Seed germination and embryogenesis in cotyledons*

The contamination in all the experiments was 10.1% (±5.8) being higher for mature zygotic embryo cultures. Germination varied from 22.7 to 66.7% at the $3^{rd}$ month of culture reaching 71.4%, 5 months after culture initiation. Direct somatic embryogenesis in cotyledons was observed 3 months after culture initiation on 30.4% of the explants. The number of adventitious embryos formed per embryogenic cotyledon varied from 1 to 20. The somatic embryos differentiated preferentially from entire or sectioned cotyledons isolated from the germinating mature zygotic embryo. Cotyledons with somatic embryos were transferred to MS28 medium for further somatic embryo development and germination. The seedlings were vigorous and had thick dark green leaves. They were subcultured to MS28 medium when they reached 2-4 cm height. Both seed germination and the development of cotyledon-derived somatic embryos were less efficient on media with growth regulators (data not shown). Similar results were reported by Vieitez and Barciela (1990).

*Leaf response: callus, direct embryo and root formation*

The physiological state of *C. japonica* shoot cultures clearly affected the leaf response independently of shoot culture origin (seeds or axillary buds). For seed-derived shoot cultures, the percentage of responding leaves (randomly selected) varied from 12.5 to 100% depending on the number of subcultures of the shoot cultures (4, 8, and 10-15 ) and on subculture interval (12, 16, and 20 weeks) (Fig. 1). The use of the 1st, 2nd and 3rd leaves, instead of all leaves of the shoot, significantly increased the leaf response after the auxin treatment (Fig. 1). Identical results were obtained using Clone Santiago and shoot cultures established from axillary buds. For optimal leaf response, shoots must be subcultured regularly, and 1st to 3rd leaves from shoots subcultured every 12 weeks should be used.

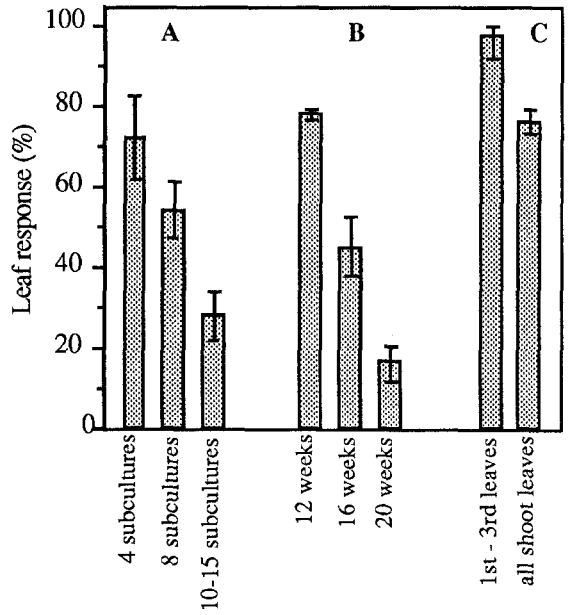
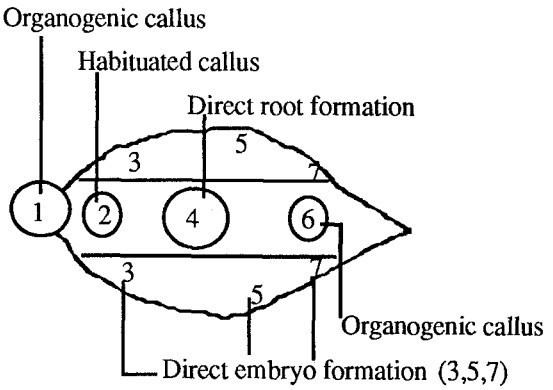



Fig. 1. Effect of the number of subcultures (A), of subculture interval (B) and of leaf age (C) on leaf response (callus, embryo and root formation) in *Camellia japonica* L., 4 weeks after culture initiation. Leaves were isolated from seed-derived shoot cultures of *C. japonica* cv. Elegans. A, Leaves from shoots subcultured 4, 8, and 10-15 times, isolated 16 weeks after the last subculture. B, Leaves from shoot cultures established 2 years before, subcultured with an interval of 12, 16, and 20 weeks. C, First (1st), second (2nd) and third (3rd) leaves and all the leaves from shoot cultures established 2 years before and subcultured every 12 weeks.

Entire leaves were used as explants because it was verified that under the culture conditions described, and unless leaves from shoots micropropagated for one year were used, leaf sectioning (in 2 or 3 portions) after auxin treatment increased browning from 0 up to 83.3% (±3.6) and prevented a morphogenic response.

Leaf response was observed with the naked eye 4 weeks after the auxin treatment. Callus formation on the leaf basis was observed first. Direct formation of somatic embryos and roots was observed one week later. The *in vitro* response (callus, embryo and root formation) was strikingly restricted to certain regions within each leaf and was independent of shoot culture origin. According to the *in vitro* response obtained 6 weeks after the auxin treatment, seven different leaf regions (lr, 1-7) were defined within a leaf of *C. japonica* (Scheme 1). Callus formation on leaf basis (lr 1) occurred in 96.5% (±4.1) of the explants cultured on solid medium and on 100% of the explants cultured in liquid medium. Callus, which frequently seemed to consist of conglomerates of globular structures (Fig. 2, A), was in fact masses of globular callus and root primordia. These calluses developed roots even while still attached to the leaf (Fig. 2, A). When transferred to callus induction medium (MS6) (Pedroso-Ubach 1991) and subcultured every 6 weeks, sustained proliferation and maintenance of rhizogenic capacity was achieved. These leaf basis-derived calluses could be induced to regenerate plants, via shoot bud induction, by transfer to MS28 medium or an other micropropagation medium (MS with cytokinin at 2-4 mg/L and auxin at 0.2-2.5 mg/L) followed by *in vitro* rooting and acclimatization (Pedroso-Ubach 1991). Callus formation was also observed on other leaf regions but always only on the midrib of the leaf. Callus was initiated on the leaf region above the leaf




Scheme 1. Different leaf regions (lr, 1-7) considered within a leaf of *Camellia japonica* L. according to the *in vitro* response obtained 6 weeks after the auxin treatment, independently of shoot culture origin.

basis (lr2) and also near the leaf tip (lr 6). The calluses formed were separated from the leaf region where they

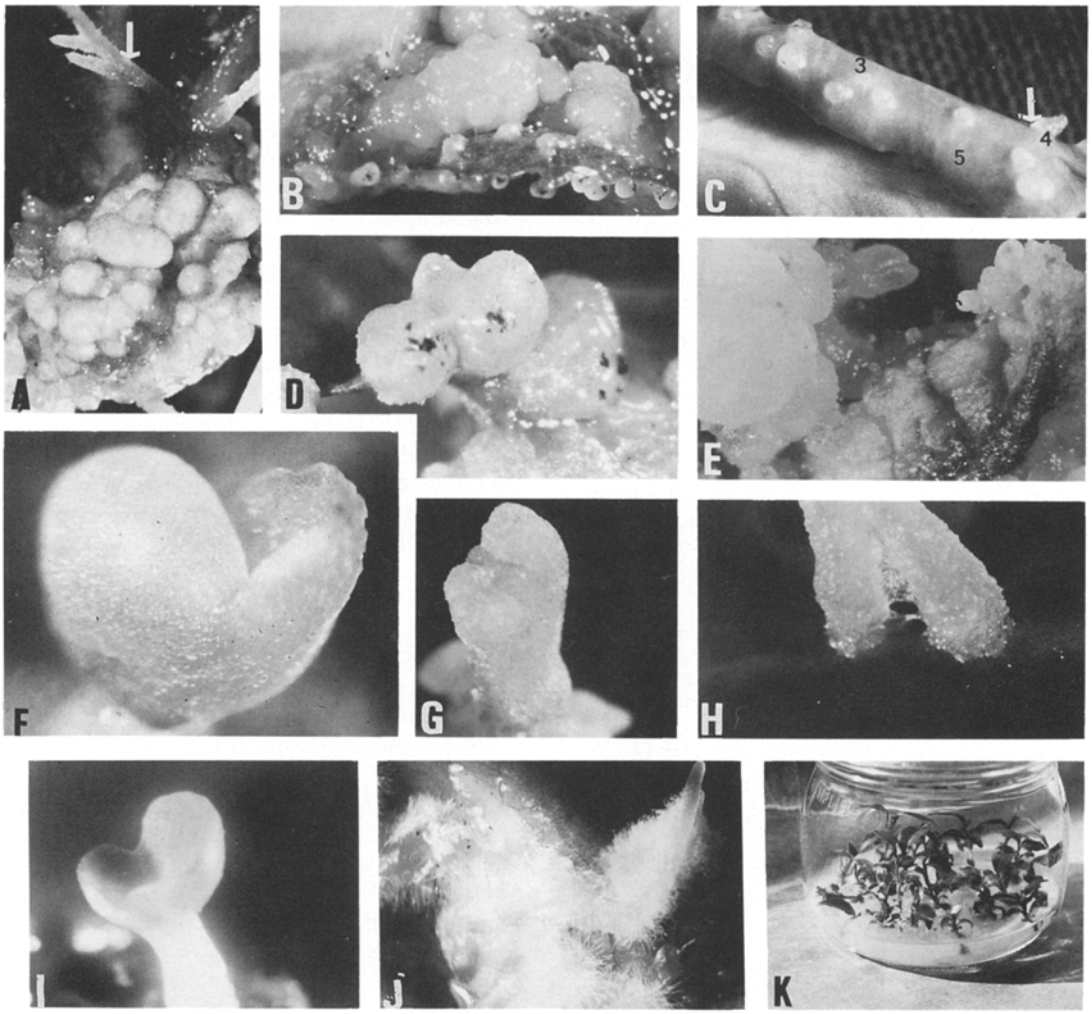



Fig. 2. Organogenic calluses and direct somatic embryo and root formation in leaves from axillary bud-derived shoot cultures of *Camellia japonica* cv. Elegans. (A). Organogenic calluses formation in lr 1 (see Scheme 1). Note root formation in the callus (arrow). (B). callus formation from the midrib in lr 2. (C). Direct globular embryo and root formation (arrow) in lr 3, 5 and 4, respectively, in a leaf cultured on diluted Murashige and Skoog modified basal medium without growth regulators (MS/2-25), 2 weeks after induction treatment. (D,E,F,G,I) different stages of direct embryo formation in lr 5 and 7. (H). Aspect of root basis and of the ruptured epidermis upon direct root formation in lr 4 (see Fig. 2C), 4 weeks after induction. (J) Roots developed in lr 4, 6 weeks after induction. (K). Shoot culture obtained from direct embryogenesis in leaves of *Camellia japonica* L. established and maintained on Murashige and Skoog modified basal medium with 1 mg/L BA and 0.1 mg/L IBA (MS28 medium).

had initiated and were established separately in culture. The calluses from leaf region six (lr 6) when transferred to micropropagation media produced shoots. So, both the calluses from lr 6 and lr 1 were organogenic. Contrary to these calluses, those from lr 2, less compact and opaque, were able to proliferate on medium without
growth regulators. For this reason they were designated habituated callus (Fig. 2, B). All the culture media and treatments tested (unpublished results) did not induce organogenesis and embryogenesis from these calluses; they seemed to be non-morphogenic. Direct somatic embryo formation only occurred in the marginal leaf regions (lr 3, 5, 7) whereas direct root formation only occurred in the midrib of the leaf slightly above the median region (lr 4) (Fig. 2, C). Both the somatic embryos and roots obtained were initiated inside the leaf without callus formation, emerged through rupture of epidermis (Fig. 2C, H), and developed normally (Fig. 2,D-J). Microscopic observation of hand made leaf sections showed that somatic embryos originated from the subepidermal parenchyma cells whereas roots originated from cells near the vascular bundles. Globular embryo formation was almost synchronous but further embryo development was

asynchronous. Several stages of embryo development from globular to cotyledonary could be observed on the same leaf. The embryos detached easily from the leaf and were frequently observed floating in liquid medium. Direct embryogenesis occurred on 11.1 to 100% of the cultured leaves being higher in leaves isolated from seed-derived shoot cultures. Higher values were obtained when $1^{st}$, $2^{nd}$ and $3^{rd}$ leaves of shoots subcultured regularly less than 8 times were used. Direct root formation on leaf region four (lr 4) occurred on 1 to 57.1% of the cultured leaves. No plant regeneration from these roots was obtained until now.

Control experiments showed that auxin treatment was indispensable for direct embryo formation from leaves cultured in MS/2-25 medium. However, direct embryo formation in leaf region three (lr 3) was already obtained in a low frequency when leaves were cultured in the presence of 2,4-D (Pedroso and Pais 1992). The auxin treatment increased direct embryo formation in all the marginal leaf regions in a reproducible way.

Leaves from shoots micropropagated *in vitro* for one year, subjected to the same auxin treatment and sectioned transversely in 5 to 9 slices responded in the same way as entire leaves forming embryos, roots and callus on the expected leaf regions. However, formation of calluses was observed at the cutting sites.

*Regeneration via direct embryogenesis*

Plant regeneration via the production of somatic embryos was developed as a more efficient alternative route as compared to regeneration from leaf-derived callus. Somatic embryo germination varied from 18 to 43.6%. The non-synchronous embryo development was responsible for the decrease of germination efficiency because when the embryos were transferred to MS28 medium, many of them were not at the late cotyledonary stage. Secondary embryogenesis was frequent, doubling somatic embryo number in 4 to 6 weeks. The multiplication rate of shoot cultures obtained from direct embryogenesis in leaves (Fig. 2, K) was identical to that obtained for other established clones (seed-derived clones).

At present the factors conditioning the morphogenic response on defined regions of the leaves are not understood. In all the leaves observed cell and leaf-region origin remained constant for embryos, roots and callus (morphogenic and habituated). Presently callus proliferation on lr 2 and lr 6 has been reduced by reducing the culture initiation on MS/2-25 medium to a maximum of six weeks preventing callus proliferation around the embryos and the roots as shown on Fig. 2B, D, E. The compartition represented on Scheme 1 is thus fully achieved. Results identical to those reported above were obtained using shoot cultures established from plant material harvested in Parque de Monserrate (Serra de Sintra, Portugal) This culture system offers the possibility of studying the expression of competence for direct embryogenesis and rhizogenesis in specific regions within a single leaf explant. Previous studies on ultrastructural and distribution changes of chemical elements during the induction of morphogenesis in this culture system have shown that the induction of embryogenesis is associated with the appearance of a callose-like material and the increase in Na, Ca, K, P, Fe, and S in the induced cells (Pedroso and Pais 1992). The present as well as the published results (Pedroso-Ubach 1991) seem to indicate that *Camellia japonica* can be a good model to study the factors controlling somatic embryogenesis and rhizogenesis.

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## References

Kato M (1986) Jpn. J. Breed. 36: 31-38.

Murashige T, Skoog F (1962) Physiol. Plant. 15: 473-497.

Pedroso MC, Pais MS (1992) Plant Sci. 87: 99-108.

Pedroso-Ubach MC (1991) Contribuição para a preservação e o melhoramento de *Camellia japonica* L. Masters Thesis ( Engl. abst.). Faculdade de Ciências de Lisboa, 23-48; 89-101; 131-144.

Vieitez AM, Barciela J (1990) Plant Cell Tis. Org. Cult. 21: 267-274.

Vieitez AM, San-José C, Vieitez FJ, Ballester A (1991) J. Amer. Soc. Hort. Sci. 116: 753-757.