Direct embryo formation in leaves of Camellia japonica L.

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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; Vicitez et Barciela 1990), roots (Vicitez 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. Vicitez 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.

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Culture conditions

Unless otherwise stated, the culture medium used was the basal medium (macro and micro nutrients 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 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 μE.m⁻².s⁻¹) provided by cool fluorescent lamps (Grolux Sylvania). Glass culture vessels (300 cm³) 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 cm³) 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 μE.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 1st, 2nd and 3rd leaves and on cultures of 1st to the 9th or 10th 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 3rd 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 Viciez and Barciela (1990).