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Induction of somatic embryogenesis in *Cynara cardunculus* L. (Compositae)

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SUMMARY

This report describes procedures for the production of somatic embryos from cotyledons and first leaves of *in-vitro* grown seedlings of *Cynara cardunculus* L. (Compositae). Embryogenic calli were obtained after 4–5 weeks of culture on solid B_5 medium containing selected combinations and concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (KIN) or 6-benzylaminopurine (BAP). In the presence of 2,4-D at 1 mg litre^{-1} and KIN at 0·1 mg litre^{-1}, initial stages of somatic embryo development were formed on the surface of the embryogenic calli. Cotyledon explants presented a higher percentage of embryogenic induction than leaf explants. The transfer of these cultures to B_5 liquid medium containing 2,4-D (0·1, 0·5, 1 mg litre^{-1}) and zeatin (ZEA) at 1 mg litre^{-1} allowed induction of more embryos and their further development. 2,4-D at the optimum concentration of 0·1 mg litre^{-1} was necessary for embryo development. Some embryos presented abnormalities that were not prevented by the addition of abscisic acid (ABA) to the culture medium. Mature embryos when transferred to the same liquid medium lacking growth regulators showed root apex growth and greening of cotyledons. However, the germination of the embryos was still only occasional and plantlets obtained showed arrest of growth before development of leaves.

Key-words: Compositae, *Cynara cardunculus* L., somatic embryogenesis.

INTRODUCTION

Cynara cardunculus is a wild species of the arid Mediterranean areas and its major economic importance is related to the traditional manufacturing of cheese. Dried flowers when immersed in milk cause clotting due to the presence of proteases. These proteases have been extracted and purified (Heimgartner *et al.* 1990). *C. cardunculus* can be used as cattle feed and is a potential source of vegetable oil and of fibre for paper paste.

The availability of an *in-vitro* regeneration method may open the way for mass-propagation, but is also a prerequisite for the application of various biotechnological techniques, such as the use of genetic engineering to obtain transgenic plants. For this purpose somatic embryogenesis is particularly advantageous because this process enables us to regenerate a whole plant from a single cell. Pro-embryos can also be

directly modified through microinjection or particle bombardment because they exist as independent masses that can readily be manipulated. To our knowledge, somatic embryogenesis in the genus *Cynara* has not been achieved yet. The globe artichoke (*C. scolymus*) has been widely studied but regeneration from somatic tissues has been accomplished only via organogenesis (Devos *et al.* 1975; Ancora *et al.* 1979; Fortunato *et al.* 1979; Ordas *et al.* 1990).

In the present study the response of cotyledons and first leaves excised from *in-vitro* grown seedlings was analysed with respect to callus initiation and embryogenesis.

MATERIALS AND METHODS

Plant material

Mature seeds of *Cynara cardunculus* L. were obtained from plants growing spontaneously at Monte Álamo (Baixo Alentejo). Seeds were dipped for 1–2 h in water containing a few drops of commercial detergent and then surface sterilized in 10% calcium hypochlorite for 40 min. Afterwards they were rinsed four times with sterile water and placed in test tubes (30 × 135 mm) containing 10 ml of Schulz (1981) medium solidified with 7 g litre^{-1} agar. Germination occurred after 4–6 days in darkness at 23 ± 2°C. One week following germination some of the seedlings were transferred to a 16-h photoperiod with a light intensity of 31·4 μE m^{-2} s^{-1} for development of leaves.

Callus induction

Cotyledons approximately 15 mm in length were excised from 6-day old seedlings grown in darkness and sectioned into three parts (proximal, median and distal). The first leaves from seedlings grown for 7 days under 16 h light conditions were also removed and cut into two transverse segments. Explants were placed flat on top of solid B_5 medium (Gamborg *et al.* 1968) supplemented with 2,4-D (1, 2, 3 mg litre^{-1}) or naphthalenoacetic acid (NAA-1, 2 mg litre^{-1}), alone or in combination with KIN or BAP (0·1 mg litre^{-1}). All media contained sucrose at 30 g litre^{-1}. The pH was adjusted to 5·6 before addition of 8 g litre^{-1} agar. Explants were inoculated in test tubes, one per tube, and maintained in darkness.

Somatic embryo development

Embryogenic calli obtained on medium with 1 mg litre^{-1} 2,4-D and 0·1 mg litre^{-1} KIN were transferred, after 5–7 weeks, to the same solidified basal medium without growth regulators or with BAP, KIN or ZEA (0·1 mg litre^{-1}) alone or in combination with 2,4-D (0·1, 0·5, 1 mg litre^{-1}). Identical hormonal supplementations were tested for somatic embryo development in liquid medium. The effect of ABA at 1 mg litre^{-1}, in addition to 2,4-D (0·1 mg litre^{-1}) and ZEA (1 mg litre^{-1}), was also tested for liquid cultures. Induced callus pieces were inoculated in 100-ml Erlenmeyer flasks, 8 per flask, containing 20 ml of medium and incubated at 140 rpm. Subcultures were performed every 2–3 weeks. Both cultures on solid and in liquid medium were maintained in darkness. Mature embryos obtained in liquid conditions were transferred to medium lacking growth regulators and kept under a 16-h photoperiod (31 μE m^{-2} s^{-1}).

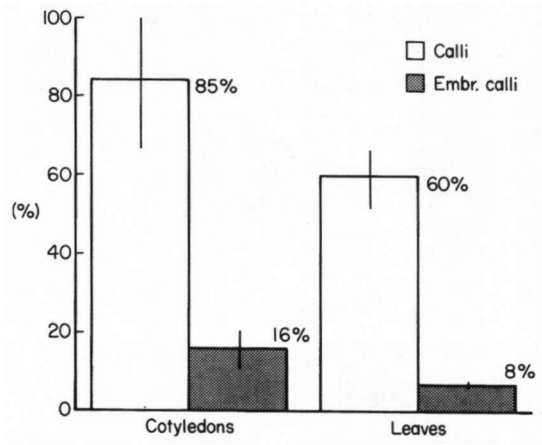

Fig. 1. Callus induction and embryogenic response in cotyledons and leaves after 5 weeks of culture on B_5 medium (1 mg litre^{-1} 2,4-D and 0·1 mg litre^{-1} KIN). Data are mean values from three assays ± SD.

Scanning electron microscopy

Selected embryogenic calli (grown in medium containing 1 mg litre^{-1} 2,4-D and 0·1 mg litre^{-1} KIN) were fixed in FAA (Acetic acid/formaldehyde 30%/ethanol 70%, 1:1:18, v/v) and dehydrated in an ethanol series (70–100%) for observations using SEM (Scanning Electron Microscopy). Samples were critical-point dried in CO_2, coated with gold and scanned with an electron microscope (JEOL JSM T220).

RESULTS

Callus induction

Ten days after culture initiation most of the cotyledon segments had formed calli (Fig. 1). A fast growing callus tissue was initially soft and translucent but after 2–3 weeks turned brown and vitrified. By this time new proliferation areas could be observed nearby the initially formed callus tissue. These areas were easily distinguished due to their compactness and yellow colour (Fig. 2a) and were considered as embryogenic calli. Leaf segments displayed similar behaviour but presented a lower embryogenic competence in identical culture conditions (Fig. 1). The percentages of induction of embryogenic callus were 16% and 7% in cotyledon and leaf explants, respectively. However, distinct cotyledon segments had different reactions. Over 85% of the cotyledon-derived embryogenic calli appeared in proximal and median segments (results not shown).

All hormonal combinations tested for callus induction were able to induce soft, translucent calli (Table 1). Formation of non-embryogenic callus was observed in 96%–100% of the explants cultured on media containing 2,4-D, but this percentage decreased to 80% in the presence of NAA. Embryogenic calli were obtained only when 2,4-D was used as auxin. Embryogenic induction was possible using 2,4-D as the sole growth regulator, but the presence of KIN at 0·1 mg litre^{-1} significantly (P=0·05) increased the frequency of the induction. BAP in combination with 2,4-D had no effect on the induction process. The highest percentage (23%) of embryogenic callus formation

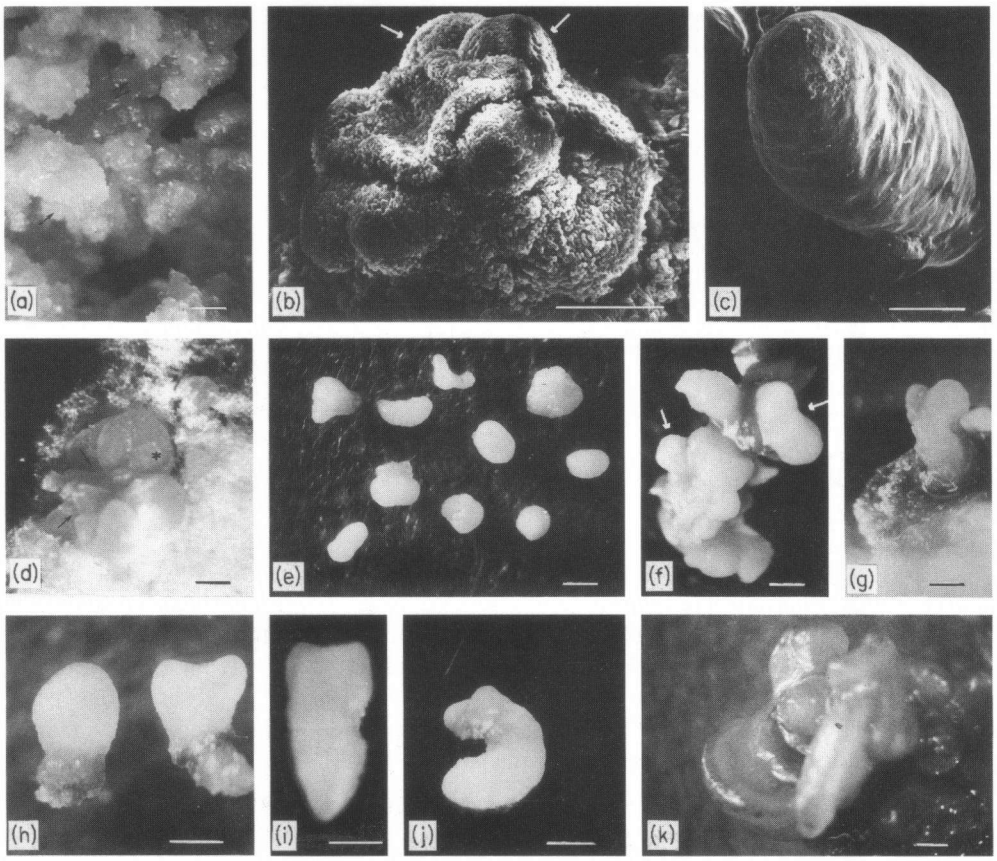

Fig. 2. (a) Cotyledon-derived callus with non-embryogenic (arrow) and embryogenic (double arrow) zones. (b), (c) Scanning electron micrographs. (b) Compact group of pro-embryogenic structures on the callus surface. (c) Pro-embryogenic structure emerging isolated. (d) Group of somatic embryos at various developmental stages, including early cotyledonary stage (asterisk), formed on embryogenic callus. (e)–(k) Cultures in liquid medium. (e) Embryoids at initial stages of development. (f) Embryoids that remained attached to the cotyledon explant. (g) Early cotyledonary somatic embryo. (h) Globular and heart shape stages. (i) Torpedo stage. (j) Initiation of root apex growth. (k) Mature embryos with large cotyledons. Scale bars: 1 mm for (a) and (d)–(k); 500 μm for (b); 50 μm for (c).

was reached with 2,4-D at 1 mg litre^{-1} and KIN at 0·1 mg litre^{-1}. For a 2,4-D concentration of 2 mg litre^{-1} a slightly smaller percentage (20%) was obtained but there was an increased browning of the explants. Higher concentrations of this hormone did not promote an embryogenic response.

Scanning electron microscopy of selected embryogenic calli obtained on 1 mg litre^{-1} 2,4-D and 0·1 mg litre^{-1} KIN showed the presence of many structures emerging from the surface of the compact callus mass (Fig. 2b). These structures corresponded to groups of pro-embryos although they occasionally emerged singly (Fig. 2c). After 5–7 weeks of culture on the same medium, some of the explants presented on the surface of the embryogenic callus globular-shaped embryos appearing sporadically fused together. Other developmental stages, such as the early cotyledonary stage, could also be observed (Fig. 2d). A range of 2–27 somatic embryos per embryogenic explant was obtained on the induction medium.

Table 1. Effect of several hormonal combinations (mg litre^{-1}) in cotyledons of *Cynara cardunculus* after 6 weeks on B_5 medium

	2,4-D (1)	2,4-D (1) BAP (0·1)	2,4-D (1) KIN (0·1)	NAA (1) KIN (0·1)	NAA (2) BAP (2)	2,4-D (2) KIN (0·1)	2,4-D (3) KIN (0·1)
No. of explants	25	14	25	16	15	20	16
Explants with callus (%)	24 (96)	14 (100)	25 (100)	13* (81)	12* (80)	20 (100)	16 (100)
Explants with embryo callus (%)	2* (7)	1* (7)	6 (23)	0	0	4 (20)	0

Data are mean values from three assays. Two-way contingency tables and the χ^2 test were used to test the significance of observed differences.
*Significantly lower (P=0·05) than with 1 mg litre^{-1} 2,4-D+0·1 mg litre^{-1} KIN.

Somatic embryo development

When embryogenic callus cultures were transferred to solidified medium no further embryogenic development was achieved, regardless of the hormonal composition. Callus proliferation occurred at the surface of the embryoids and most of the cultures turned brown. Cultures either on solid or in liquid medium without hormones promoted root differentiation (results not shown).

The addition of 2,4-D (0·1–1 mg litre^{-1}) and ZEA (1 mg litre^{-1}) to the liquid culture medium induced the continued formation of globular embryos and promoted development into heart and torpedo stages. Some of the embryos became free in the medium (Fig. 2e), but others remained attached to the callus mass (Fig. 2f). The mean number of embryogenic structures in the suspension culture per Erlenmeyer flask was 23 ± 12·1 or 32 ± 13·3 in media containing ZEA (1 mg litre^{-1}) and 2,4-D (0·1 mg litre^{-1}) or 2,4-D (1 mg litre^{-1}), respectively. The use of BAP or KIN instead of ZEA resulted in progressive browning of the cultures and no embryogenic development occurred. The same happened in media without 2,4-D.

After the second subculture, embryogenic structures maintained in medium containing 1 mg litre^{-1} 2,4-D and ZEA started to dedifferentiate and formed callus masses. If the concentration of 2,4-D was 0·1 mg litre^{-1} different stages of embryogenesis were simultaneously observed: globular, heart, torpedo, and, less frequently, embryos at an early cotyledonary stage (Figs 2g–i). However, a high number of abnormal structures were also present. Furthermore, the root apex often developed before the embryos reached a mature stage (Fig. 2j). The addition of ABA did not prevent these abnormalities.

After a third subculture a few embryos reached maturation and showed a great increase in cotyledon size (Fig. 2k), but their transfer to medium without hormones only occasionally promoted germination. Plantlets obtained stopped growing before development of leaves and eventually necrosed.

DISCUSSION

The induction of somatic embryogenesis in *C. cardunculus* was dependent on the type of explant used. Cotyledons were a better source of embryogenic callus than leaves. However, distinct cotyledon zones responded differently to the same culture conditions.

This kind of behaviour is frequently observed not only in cotyledons of various plant species (Vieitez & Barciela 1990) but also in leaves (Vasil 1987; Pedroso & Pais 1992) and it is probably related to ion gradients or to the endogenous pools of growth regulators which cause a gradient of embryogenic competence in the explants.

The hormonal supplementation also influenced the embryogenic induction in *C. cardunculus*. 2,4-D was essential for this process. The use of NAA or any other auxin (unpublished results) always resulted in the formation of non-embryogenic callus. In fact, 2,4-D is the most widely used auxin for embryogenic cultures (Ammirato 1983), especially in other Compositae such as *Helianthus annuus* (Prado & Berville 1990). However its efficiency was dependent upon the concentration used. The most efficient concentration was 1 mg litre^{-1}. For the higher levels, the production of phenolic compounds seemed to be stimulated and cultures tended to turn brown. At 3 mg litre^{-1}, embryogenic induction was even inhibited. This inhibitory effect has been reported for other species (Thomas & Scott 1985; Linacero & Vazquez 1990; Nadel *et al.* 1990). According to Li *et al.* (1985) concentrations of 2,4-D higher than 2 mg litre^{-1} were avoided in cultures of *Glycine max* to prevent the loss of embryogenic competence caused by high levels of this hormone. Cytokinins were not determinant for embryogenic induction in *C. cardunculus* but KIN significantly increased the frequency of the induction. Studies conducted in anise cells (*Pimpinella anisum*, Ernest & Oesterhelt 1984) led to the conclusion that cytokinins were not involved in embryo development directly, rather they showed a stimulating effect on cell division, resulting in an increased number of cells and, therefore, more embryos in culture. The results obtained for *C. cardunculus* indicate that KIN might have a similar mode of action in these cultures.

Development of somatic embryos of *C. cardunculus* was strongly influenced by physical culture conditions. Callus proliferation at the surface of embryos always occurred if cultures were maintained on solid medium, independently of the hormonal supplementation. The transfer to a liquid medium allowed further development into later stages of embryogenesis. Identical observations were reported for somatic embryos of sunflower (Prado & Berville 1990). Embryogenic explants cultured on solidified medium formed calli on still active meristematic regions at the embryo surface. The cells dividing actively did not detach from the embryos, causing a callus proliferation and inhibiting further embryo development. In liquid medium these cells were continuously removed preventing callus formation at the surface of the embryos. Liquid culture was therefore determinant for development to proceed.

2,4-D was important not only for the induction but also for the development of somatic embryos. Its presence, at a concentration of 0·1 mg litre^{-1} together with ZEA (1 mg litre^{-1}), promoted embryo development, the more advanced stages of embryogenesis having been observed under these conditions. However a high number of abnormal embryo-like structures were also formed. ABA, which is often used as an effective compound in promoting maturation, avoiding at the same time the formation of anomalous proliferation (Ammirato 1974), had no effect on the embryo development of *C. cardunculus*. These abnormalities are probably related to a long exposure to 2,4-D.

The rare occurrence of germination and the arrest of growth before development of leaves may be due either to inadequate external conditions or to internal factors. It is possible that the normal developmental programme had been distorted by previous culture conditions. Long exposure to 2,4-D is probably responsible for the low percentage of embryos that grow to maturity. Assays are under way to achieve systematic germination of the embryos and normal plantlet growth.

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