PERMANENT SOMATIC EMBRYOGENESIS WITH L-PPT IN BRASSICA NAPUS L. CULTURES

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INTRODUCTION

Somatic embryogenesis in tissue culture was discovered at the beginning of the sixties in carrot (Steward et al. 1958; Reinert 1959; Halperin and Wetherall, 1964). Somatic embryos have been investigated in detail in many species which was reviewed by D'Amirato (1989). Thomas et al. (1976) observed secondary embryogenesis in *Brassica napus*. Schenk (1983) and Kohlenbach (1983) maintained secondary embryogenic cultures of *B. napus* for some time on hormone free medium. We report here on a system to produce unlimited amounts of adventitious embryos of *B. napus* in petri plates on solid medium containing L-phosphinotricin (L-PPT). These embryos can be used for further propagation or numerous tissue culture approaches ie. transformation of embryos or for regeneration of plants. The system can be used for both haploid and diploid explants.

MATERIALS AND METHODS

Two different plant regeneration systems were applied to produce permanent embryogenic cultures: regeneration from mesophyll protoplasts and from microspore-derived embryos. Both methods have been described previously (Bornman 1985; Phan et al. 1988; Oelck et al. 1991). In all experiments, cultures had been on B_5 medium (Gamborg et al. 1968) for several weeks to produce either callus (medium contained 0.5 mg/ℓ of 2,4-D, BAP and NAA for protoplasts) or embryo derived plantlets (medium devoid hormones). The microspore-derived shoots which were chopped into 1-5 pieces, developed directly into adventitious embryos, placed on regeneration medium (MS medium + 85 mg/ℓ arginine, 1 mg/ℓ BAP 200 mg/ℓ carbenicillin + 200 mg/ℓ + Claforan). Plant material was then treated with 60 mg/ℓ L-PPT (Hoechst AG, Frankfurt, Germany) for periods of four or eight weeks on MS medium (Murashige and Skoog 1962). Fresh medium supplemented with 200 mg/ℓ of carbenicillin (Sigma) and cefotaxime (Claforan® Hoechst AG, Frankfurt, Germany) which controlled the bacteria in the transformation experiments was given to the cultures either every 14 days or every four weeks. For the controls, the antibiotics were applied at the same concentrations. The explants surviving 60 mg/ℓ L-PPT were transferred onto fresh plates with the same medium and the dead portions around the green explants were removed. This selection was continued for several transfers (3-6 months) after which only tissue surviving the elevated L-PPT treatment remained. These L-PPT tolerant explants were regenerated to plants on L-PPT free medium and later again treated with L-PPT or transferred to soil.

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The stable, embryogenic L-PPT -tolerant cultures were analyzed histologically. This analysis will be described in detail (Heil, Ph.D. thesis 1991, in prep). The plant structures were embedded in Glycolmethacrylat (GMA) (Federn and O'Brien 1968) system Kulzer. Triple staining was carried out with Periodic Acid-Schiff (stains starch red), Fast Green (stains proteins green), and Toluidinblue (overpowers the green, giving the proteins a blue colour and stains middle lamella purple).

RESULTS

For both systems (protoplast and microspore derived), more than 10,000 plantlets have been regenerated in the last five years as a result of transformation experiments with Agrobacterium. This includes the controls which were never treated with bacteria. The controls were subjected to the same selection pressure with L-PPT as the supposed transformants and may account for at least 500 plants regenerated in both systems. By far, most of the transiently L-PPT tolerant explants proved to be non-transformed. Extended applications of various concentrations of L-PPT on some of these cultures resulted in the formation of prolific secondary embryogenesis. Initially, these effects were only observed as a side effect in Agrobacterium -treated transformation experiments and in the controls. Most cultures (96-99%) died with application of 60 mg/ℓ L-PPT (3 x 10-4M). The same effect was also found with equivalent application of DL-PPT and with the herbicides Basta® or Ignite®, these herbicides contain 20% DL-PPT. The cultures lost the increased PPT resistance once they were grown on an L-PPT free medium for a while. However, similar effects were also observed in cultures which were treated with lower concentrations between 5-60 mg/ℓ. We drew the general conclusion that the L-PPT concentration had to be at a sublethal dosage to show the effect of secondary embryogenesis. Depending on the sensitivity of certain tissues to L-PPT, the concentration had to be varied. Embryogenic tissue showed the highest tolerance to L-PPT. We observed an additional habitation effect which allowed to grow cultures for three years on up to 140 mg/ℓ L-PPT.

There was no difference between the diploid protoplast-derived embryogenic cultures and the haploid microspore-derived embryogenic cultures as far as the effect of L-PPT was concerned. This is of interest because the protoplast derived cultures went through a long callus phase before embryos were produced. The cultures continued to produce dark green embryos, while the tissue which did not respond died, therefore, a very strong selection for embryogenesis was carried out. All further steps of regeneration towards shoot or root elongation were inhibited by L-PPT. New embryo formation occurred as the only form of growth possible in the presence of lethal dosages of L-PPT. The tissue developed a layer structure with the older embryos on the medium, younger embryos on top of these and the youngest ones growing in the periphery or on top of each small embryogenic clump. The old embryos eventually turned brown, but the youngest embryos were always dark green. Sometimes embryos separated and fell off the tissue where they had originated from.

The histological investigation to answer, when, where, and how these embryos originated led to the conclusion that these L-PPT -induced embryos originated from single cells on the epidermal layer of the explants (Figure 1). It seems that at a competent stage, all epidermal cells possess the embryogenic capacity, however, most embryos die during development. A few embryos continue to develop and produce monopolar structures, either shoots or roots. The other pole to complete whole plantlets, develops later (Figure 2). Once some sort of a hypocotyl is formed, secondary embryogenesis starts.

DISCUSSION

This investigation reports the side effect observed over five years on how non-transformed tissue behaves at lethal L-PPT concentrations.

The secondary result is the description of an extremely efficient system for plant propagation by forcing tissue to either produce embryos or die.

The third finding derives from the histological investigation made possible because the L-PPT system produces completely homogenous embryogenic cultures. Abnormal embryos do occur in the cultures, but this is to be expected since somatic embryogenesis has been shown to be a stress reaction of plants (Pecan and Keller, 1989).

A large number of epidermal cells have the capacity to undergo encapsulation, division, and after a globular stage, develop a monopolar structure. Subsequently, the tissue underneath shows vascular structures as a result of the strong sink effect of the growing meristematic region. The vascular initiation results finally in the formation of a second pole of the structure which further develops into the root. As soon as this stage is reached, the structure elongates to develop cotyledons which leads to a hypocotyl region similar to normal zygotic seedlings. Under PPT influence, this region will already be programmed to continue with the embryogenic pathway. The final product of this cycle are embryos which are normal compared to zygotic or microspore-derived embryos formed *in vitro*.

Before this dipolar embryo stage is finally reached, the structures should be called embryoids according to Sihachak and Ducreux (1987) who described similar structures on *Solanum melongena* protoplast-derived callus.

It is difficult to understand which function the PPT might have on controlling differentiation. Most likely PPT does not activate, but only limits differentiation. The tissue was probably determined before the PPT application to undergo somatic embryogenesis. 2,4-D is well-known to cause such effects. The L-PPT only limits the further development of normal roots and shoots, but still allows the formation of young embryos. The reason might be the increased content of glutamine synthase together with other amino acids in such embryos compared to other plant tissue. Investigations are underway. Glutamine synthase inhibition is the primary effect of L-PPT and it follows that tissue with endogenously higher contents should show PPT resistance. Furthermore it has been shown that higher glutamine synthase activity can induce PPT resistance (Donn et al. 1987).

We have also maintained a PPT resistant alfalfa (Medicago sativa L.) culture for three years from which has shown continuous embryogenesis on a level of 20 mg/ℓ results L-PPT. D'Hallnin et al. (1990) reported the recovery of a large number of somatic embryos from a substrate containing 10 mg/ℓ L-PPT as a selective agent in transformation experiments. They assumed that at a certain stage of development the embryos are less sensitive to phosphinotricin. Most of their regenerated plantlets coming from these embryogenic cultures were non-transformed. We observed the same in B. napus.

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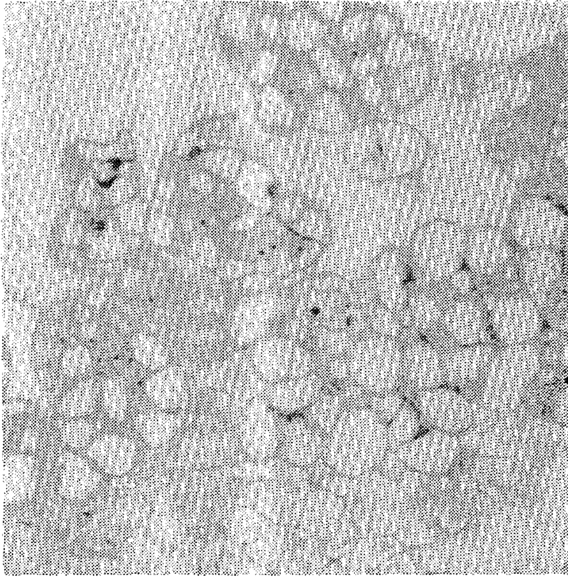

Figure 1: Embryo formation from epidermal layer

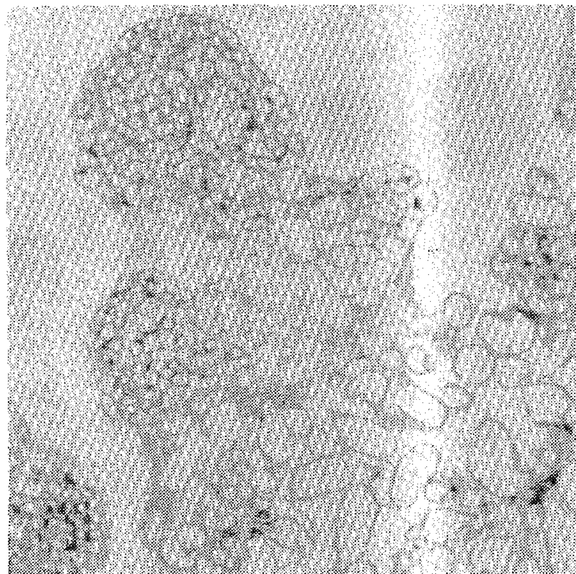

Figure 2: Advanced embryo development