# PLANT REGENERATION FROM CELL SUSPENSION AND PROTOPLAST CULTURES OF CAPSELLA BURSA-PASTORIS (L.) MEDIC.

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## INTRODUCTION

Capsella bursa-pastoris (shepherd's purse) originates from the Mediterranean region, and is found on wastelands as well as on cultivated fields throughout the world, except Polynesia (British herbal pharmacopoeia, 1983). In addition to being cold tolerant and having a short life-cycle, shepherd's purse is not attacked by flea beetles, which represent major pests of Cruciferous crop species. C. bursa-pastoris and crop species such as Brassica napus, B. campestris or B. juncea belong to different tribes and are sexually incompatible, so that it is not possible to introduce Capsella's traits into Cruciferous crops by the production of sexual hybrids. On the other hand, agronomically interesting traits may be transferred through somatic hybridization, or by protoplast fusion. Zilkah and Gressel (1977) reported callus development from shepherd's purse whole seed, hypocotyl, and cotyledon explants, from which shoots and roots differentiated, but neither cell suspension initiation, nor protoplast isolation have yet been reported for this species. We describe, for the first time, the initiation and maintenance of embryogenic cell suspension cultures of C. bursa-pastoris, and the isolation of cell suspension-derived protoplasts from which embryogenic calli developed. Since Brassica protoplast culture systems have been widely studied (e.g. Pelletier et al., 1983; Glimelius et al., 1984, Klimaszewska and Keller, 1985, 1986, 1987; Toriyama, 1987; Kirti and Chopra, 1990), this work will allow the use of shepherd's purse as a gene source for Brassica genetic improvement, through protoplast fusion.

## MATERIALS AND METHODS

Cell Suspension Initiation and Maintenance.

Callus cultures were initiated from leaves of three week old in vitro grown plantlets, cultured on agar-solidified MS medium (Murashige and Skoog, 1962), with 0.5 to 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Cell suspensions were initiated by placing approximately 2 g of callus tissue into 30-35 ml of liquid MS medium , supplemented with 2 mg/l 2,4-D. The suspensions were transferred weekly, and kept on gyratory shakers (140 rpm) at 25°C under continuous fluorescent light. These suspensions were three years old at the time of experimentation. More recently, cell suspensions were initiated directly from leaves, which were finely lacerated and cultured in liquid MS medium, 2% sucrose, 2 mg/l 2,4-D. The cultures were blended three times (in a Wareing blender) for a few seconds, at monthly intervals. These suspension cultures were 6 months old when used.

Regeneration on Solidified Media.

The cell suspension culture medium was filtered through a 44 um nylon mesh. Cell aggregates of 1.0 to 2.0 mm diameter, composed of several cell clusters and isolated cells, were transferred onto regeneration medium, solidified with 1% agarose (SeaPlaque) or 0.2% gelrite. Various hormones and solidifying agents were compared for their effect on embryogenesis and organogenesis. Calli were transferred onto fresh medium after 4 weeks, and results were recorded after two months. Regenerated shoots and embryos were transferred onto hormone-free $B_5$ medium for root initiation. Plantlets were planted in Jiffy 7 peat pellets in a mist chamber for 2 weeks, then transferred to soil in a growth cabinet, 20/15°C (day/night) temperature, 16 h photoperiod.

Protoplast Isolation and Culture.

Approximately 0.5 g (fresh weight) of cells, collected on 44 um nylon mesh, were digested in 15 ml of enzyme solution (2% macerozyme R10, 1% cellulase "Onozuka" RS (Yakult Honsha, Tokyo), 0.05% pectolyase Y-23 (Seishin Pharmaceuticals, Tokyo), 0.4 M mannitol, pH 5.8), and incubated at 28°C on a reciprocating shaker (50 rpm) for 3 to 6 h in the dark. Protoplasts were filtered and washed by centrifugation in a salt solution (0.2% $CaCl_2.2H_2O$., 2.5% KCl, pH 6.9). 1.5 ml of protoplast suspension ($10^5$ protoplasts/ml) were plated in culture medium per 60X15 mm Petri dish, over 2 ml of culture medium solidified with 0.4% agarose Seaplaque (FMC, Rockland). Dishes were incubated at 25°C in the dark for 7 days. Routinely, culture media were B (Pelletier et al., 1983) or 8p (Kao and Michayluk, 1974). After 7 days, protoplasts were fed with 0.75 ml C medium (Pelletier et al., 1983), or modified 8p (Glimelius et al., 1984), and placed under diffuse light; they were fed again after three weeks with 1 ml of D (Pelletier et al., 1983), and placed under bright light. Colonies were transferred onto regeneration media once they had reached 0.5-2.0 mm diameter.

## RESULTS AND DISCUSSION

Cell Suspension Initiation and Maintenance

Unless mentioned otherwise, most experiments were carried out with the 3 year old cell suspensions. At the time of experimentation, this suspension was fine, with most aggregates containing from 20 to 100 cells. In one week, the packed volume increased from 1.75 ml (1 g fresh weight inoculum) to 8.85 ml. Most cells were rapidly dividing, isodiametric, had a dense cytoplasm, and small vacuoles. Xylem differentiation occurred frequently, but no organogenesis was observed in the presence of 2,4-D.

Regeneration on Solidified Media

Excellent embryo formation was triggered on hormone-free solidified medium, or by a combination of 1.0 mg/l NAA and 0.5 mg/l BAP (Table 1). Rinsing the cells before plating, in order to remove as much 2,4-D as possible did not affect embryogenesis, which might suggest that a supply of exogenous 2,4-D was not required for embryogenesis at that stage. Both 1 mg/l NAA and 0.5 mg/l BAP reduced the frequency of embryogenesis, and were therefore considered slightly inhibitory. In contrast with the findings of Sikdar et al. (1990), with Diplotaxis muralis protoplasts, we found GA3 to be inhibitory to embryogenesis. Cell clumps and root meristems previously cultured in hormone-free liquid media for three

weeks did not respond at all in terms of embryo production. Although thousands of embryos developed, only a few were morphologically normal. Secondary embryogenesis, or the development of embryos on the primary embryos, was a common problem. Fused, multiple cotyledons, premature elongation, or swelling and vitrification occurred often, and complete embryo maturation was usually not achieved. Necrosis of the suspensor was commonly observed. As a result, roots did not develop on most of the embryos upon transfer to rooting medium, despite their clear bipolar nature and the presence of well defined root apices. However, non-vitrified and very leafy material was readily rooted, and plants were regenerated, transferred to soil, and acclimated to growth chamber conditions. Regenerants showed typical lanceolate leaves with basal acute auricles. Abnormal flower development was commonly observed, with aberrant numbers of petals or sepals, poor pistil and anther development. Triangular seed-pods were formed from all flowers. Plants recovered from the three year old suspensions were usually sterile. These suspensions had lost some regeneration potential and probably contained numerous abnormal aneuploid or polyploid cells. The 6 month old suspension was also plated on hormone-free solidified media, and 31.6% of the calli regenerated shoots after one month. Most plants regenerated from that suspension were morphologically normal and set seeds.

Table 1: Effect of pretreatments on somatic embryogenesis in cell colonies from suspension cultures of C. bursa-pastoris (L.), plated on various solidified media. Data were recorded after two months.

| | # colonies with embryos | | |
|---|---|---|---|
| SOLIDIFIED MEDIUM | no pretreatment | rinsed with hormone-free medium before plating | cultured in hormone free medium for 3 weeks before plating |
| hormone-free 1% agarose | 73.4 | 74.4 | 0.0 |
| hormone-free 0.2% gelrite | 74.7 | 72.7 | 0.0 |
| 1.0 mg/l NAA 0.5 mg/l BAP | 50.2 | 51.7 | 0.0 |
| 1.0 mg/l NAA 0.5 mg/l BAP 0.05 mg/l GA3 | 10.0 | 6.7 | 0.0 |

Protoplast Culture

Protoplast yields of up to $3x10^7$ /g fresh weight cell suspension were readily obtained. Some aggregates of 10 to 100 cells still remained intact, but these were eliminated by filtration through the nylon filters. Both 8p (Kao and Michayluk, 1974) and B (Pelletier et al., 1983) media promoted cell wall regeneration within 3 days, with 62% cell viability and 7% divisions after one week of culture. The effects of media additives such as 1% PEG, 0.25 g/l acid hydrolyzed casein, 2 g/l BSA, and 20 ml/l coconut milk are shown in Table 2. After one month, thousands of microcolonies developed, regardless of the media. Embryos regenerated on hormone-free solidified media, from which 4 plantlets were recovered to date. Embryogenic potential was retained in these suspension cultures for more than 3 years, as was reported for Arabidopsis thaliana cell suspensions (Gleddie, 1989; Ford, 1990) This is in contrast with B. nigra cell suspensions, that displayed a marked reduction in embryogenic potential after 50 weeks (Klimaszewska and Keller, 1986).

Table 2: Viability and division rates of suspension-derived C. bursa-pastoris protoplasts.

| MEDIUM | VIABILITY (%) | DIVISION (%) |
|---|---|---|
| NN ficoll | 55.5 | 1.6 |
| 8p | 62.0 | 7.0 |
| 8p casamino ac. | 60.0 | 3.4 |
| 8p BSA | 55.9 | 4.3 |
| 8p coco. milk | 69.0 | 6.0 |

Data were recorded after one week in culture. Additives were: 2% (w/v) ficoll, 1% (w/v) PEG, 0.25 g/l casamino acids, 2 g/l BSA, 20 ml/l coconut milk.

## CONCLUSIONS

This work reports plant regeneration from cell suspension cultures and protoplasts of C. bursa-pastoris. Wild relatives have been considered useful in providing genetic sources for improving Brassica crops (Gleba and Hoffmann, 1980; Toriyama et al., 1987; Chatterjee et al., 1988; Sikdar et al. 1990). Our ability to isolate protoplasts and regenerate plants from C. bursa-pastoris may allow the transfer of genetic traits from this species to agronomically important Brassicaceae species, through protoplast manipulation and somatic hybridization.

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