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INDIGENOUS PRODUCTION OF SYNTHETIC SEEDS IN *DAUCUS CAROTA*

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Abstract

Embryogenic callus culture of carrot (*Daucus carota* L.) was induced from the hypocotyl explants in MS medium supplemented with Casein hydrolysate, 2,4-D and BAP for somatic embryogenesis *In vitro* cell suspension cultures for high multiplication rate. Embryogenic callus was transferred to liquid basal Murashige and Skoog medium The mature somatic embryos were encapsulated in a Sodium alginate mixture to produce synthetic seeds. Sodium alginate 2% and Calcium chloride 75mM was best for encapsulation. Germination frequency of encapsulated somatic embryos was evaluated upto 10 weeks successfully.

Introduction

Carrot (*Daucus carota* L.) is a winter season crop grown for its edible storage taproots throughout the world. Somatic embyogenesis is a vegetative propagation method in which cells from seed embryos are stimulated to develop somatic embryos to produce plants. Somatic embryogenesis from cultured cells is a useful system for propagation of plant material *In vitro* and the production of synthetic seeds. Encapsulation of somatic embryos can be considered an important application of micropropagation, to improve the success of *In vitro* derived plant delivery to the field or to contribute to the synthetic seed technology. Natural seeds are usually genetically heterogeneous and much time is needed to produce seeds that are homogeneous in terms of their genes. However, if developing embryos obtained by somatic embryogenesis are used directly for propagation of plants or are encapsulated in a suitable material that promotes germination, plant propagules with the same genes as the mother plant can be obtained simply and standard laborious breeding procedures can be avoided. This process of planting can be easily mechanized and this allows the direct delivery of tissue cultured plant propagules to the field. It also provides rapid bulking up for the production of individual genetically engineered plants. Usually somatic embryogenesis occurs at low frequency and at different development stages of somatic embryos are always present in a given culture. A large number of embryos at a given stage is needed as starting material for such purposes.

According to the literature, somatic embryogenesis for a variety of plants has been achieved using a variety of media ranging from relatively dilute White's medium (White, 1963) to the more concentrated formulations of Gamborg *et al*., (1968), Schenk & Hildebrandt (1972) and Murashige & Skoog (1962). Over 70% of the successful cases used Murashige & Skoog (1962) salts or its derivatives (Evans *et al*., 1981). Of the plant growth regulators, auxin is known to be essential for the induction of somatic embryogenesis in some plant species, although 2,4-D is the most commonly used auxin. Other auxins may be required for certain species (Ammirato, 1983). For example, only NAA was found to induce somatic embryogenesis in *Solanum melongena*. For the maturation of somatic embryos however, transfer to media containing a low concentration or devoid of 2,4-D was essential (Ammirato, 1983; Cheema 1989; Van der

Valk *et al*., 1989; Komamine *et al*., 1990). In some plant species, a combination of 2,4-D or NAA with cytokinin was reported to be essential for the induction of somatic embryogenesis (Kao & Michayluk, 1981; Gingeas & Lineberger, 1989; Schuller *et al*., 1989). 2,4-D has been successfully used in a large number of somatic embryogenesis studies. It is an inhibitor for precocious embryo germination, whereby it allows embryo maturation to proceed in a more normal fashion, generally increasing the uniformaity of produced embryos and reducing the development of abnormal forms (Ammirato, 1983). Redenbaugh *et al*., (1991) suggested that maturation of alfalfa, carrot and celery somatic embryos with ABA was also critical for conversion of somatic embryos into plantlets. Therefore, attempts have been made to induce somatic embryogenesis synchronously and at high frequency in cell suspension culture.

Materials and Methods

Plant materials and growth conditions: Seeds of *Daucus carota* L., variety T-29 were surface sterilized by soaking in 70% (v/v) ethanol solution for 2 min., and then immersed in 0.1% (w/v) Mercuric chloride for 10 min., rinsed 3 times in sterilized distilled water and placed on moist filter paper in the dark at 25^0C for germination

Callus induction and establishment of cell suspension: A number of combinations of different media were assessed for callus induction and cell suspension culture. Fast growing, light green to creamy callus was established from hypocotyle (5 days after sub-culture) on MS basal medium containing 2.5mM 2,4-D, 3% Sucrose and 0.1 % Phytagel at pH 5.7 at 25^oC with a 16-hrs. photoperiod. Different media containing 2,4-D, IAA, NAA, alone or in combination with Zeatin, Kinetin and BAP were used as growth regulators. The effect of medium type was preliminary investigated from the embryogenic callus induction stage to globular embryo stage, light green to creamy, fast growing and friable callus. Once initiated, embryogenic callus was either transferred to liquid medium or maintained as stock culture by sub-culturing small piece of callus onto fresh medium after every 3 to 4 weeks.

Suspension cultures were initiated by transferring one month old embryogenic and friable callus to 50ml MS basal medium containing 25μM Adenine sulfate, 2.8μM Thiamine HCl, 575μM NaH_2PO_4, 0.1% Casein hydrolysate, 0.5μM BAP and 6μM 2-4-D in a 250ml Erlenmeyer flask and placed on a gyratory shaker with agitation at 120rpm at 25^oC in 16-hr photoperiod. Suspension cultures were sub-cultured after every 15-day for 3 to 4 sub culturing and unused cultures were discarded.

Somatic embryogenesis: Cells in Erlenmeyer flasks were sub-cultured after every 15-days. After subsequent sub-culturing, liquid medium was modified by reducing casein and growth hormones for the initiation of embryos development. For a synchronized embryos population, pro-embryos suspension was passed through a series of stainless steel sieves, first at 150μm (100 mesh) and then 75um (200 mesh) and cultures were kept on a rotary shaker.

Encapsulation of somatic embryos: Somatic embryos of carrot at late torpedo stage were encapsulated by mixing with sterilized 2% (w/v) Sodium alginate solution containing 3% sucrose. Alginate droplets dropped into 75mM Calcium chloride solution under continuous stirring were kept for 30 min., for hardening the coating of Calcium alginate formed around the embryos. The synthetic encapsulated beads were washed with 5mM $CaCl_2$ solution for one hour and collected by filtration.

Germination of artificial seeds into plantlets: Encapsulated somatic embryos were germinated on MS basal medium containing 3% Sucrose and 0.1% Phytagel and incubated at 25^{o}C with a 16 hrs., photoperiod for germination.

Results and Discussion

Embryogenic callus formation: Most important factors affecting the induction of embryogenic callus and plant regeneration through somatic embryogenesis include the explants type, media formulation and growth regulators. The most important factor that affected the callus type was the growth regulator. Depending on these conditions, light brown friable calli which were nonembrogenic, green compact with low embryogenic frequency whereas yellowish-nodular friable with high embryogenic frequency were obtained (Fig. 1).

Induction of somatic embryogenesis required a change in the fate of a somatic cell. The general observation in this study was that 1AA and IBA were ineffective, not stimulated any embryogenic callus, NAA stimulated the formation of low-frequency embryogenic callus and addition of 2,4-D was very much effective in the stimulation of embryogeinc callus (Fig. 2). Further experiments were performed in order to determine the optimum concentration of 2,4-D. Best explants source was the hypocotyle and the optimum 2,4-D concentration was 2mM with high embryogenic frequency. When concentration of 2,4-D was increased gradually up to 12μM, the browning of callus was noted with low embryogenic frequency. By this stage of investigation, it was also observed that the root explants did not produce any embryogenic callus under any condition. Consequently, it was excluded from the rest of the study.

Suspension culture: After embryogenic callus induction, cells were proliferated on both modified MS solid and liquid medium containing 1% Casein hydrolysate and 2mM 2,4-D. Cell suspension were cultured on a rotary shaker (120rpm) under the same conditions as for callus formation. In the preliminary attempts of embryo initiation, embryogenic cell suspension cultures were transferred to growth regulator free modified MS basal liquid medium. In this medium, cell clusters developed through globular-shaped into heart shaped embryos but no further. Casein hydrolysate is reported to be effective on embryo development. When pre-somatic embryos were transferred to modified MS liquid medium free of Casein hydrolysate and growth regulators, somatic embryos of torpedo-shaped appeared after two successive sub-culturing.

Synchronization of embryogenesis: The procedures for synchronizing of the carrot followed those of Giuliano *et al*., (1983). All cells and cell aggregates were passed through 150uM Sieve (100mesh) and incubated on Gyratory shaker at 25^{o}C at 120rpm. After 4 days, proembryos were passed through 75uM (200mesh) and diluted with medium as mentioned in methodology free of casein and growth hormones. Uniform cell suspension in casein and growth hormones free medium for 5 to 7 days resulted in the development of globular embryos. Addition of fresh medium routinely maintained synchrony of the cells from heart stage to torpedo-stage.

Fig. 1. Embryogenic callus formation in *Daucus carota* L.

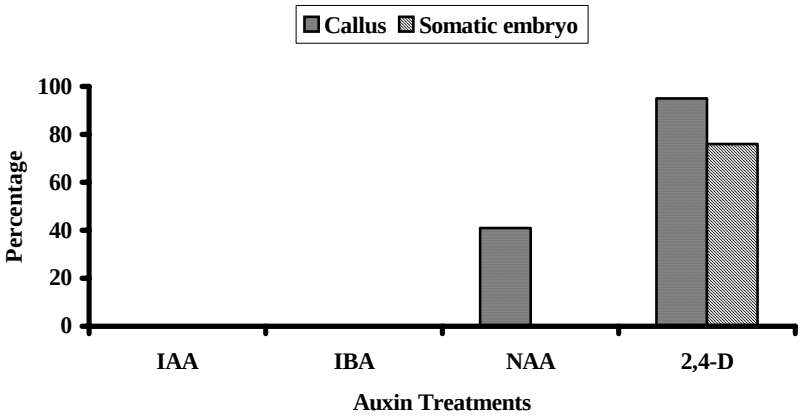

Fig. 2. Effect of auxin on callus and somatic embryo formation.
IAA: Indole-e- acetic acid, IBA: Indole-3-butyric acid, NAA: Naptholene acetic acid, 2,4-D: 2,4-dichlorophenoxy acetic acid

Encapsulation with alginate gel: Somatic embryos encapsulation seems to be one of the promising methods for sowing embryos, because encapsulation with the proper materials and structure will not only protect somatic embryos from physical damage or desiccation during the delivery or sowing process in the greenhouse, but also enable easy handling and automation.

Alginate hydrogel is frequently selected as a matrix for synthetic seed because of its features including moderate viscosity and low spinnability of the solution, low toxicity and quick gelation which is an important characteristic for the application of droplet hardening method (Rederbangh *et al*., 1993). Alginate beads were made quickly by dropping Sodium alginate solution (2%) with somatic embryos into hardening solution, 75mM Calcium chloride (droplets hardening methods). Beads (Fig. 3) were hardened for 10 to 30 min., followed by rinsing with auto-dist H_2O to remove excess Calcium ions.

Germination efficiency of synthetic seeds: Germination frequency of synthetic seeds stored at 4°C was checked upto 10 weeks (Fig. 4). Thirty seeds were grown on MS basal medium at 25°C. Germination generally occurred in 6-8 days. After 3 weeks, the germination frequency was stable. The conversion frequency was from 60% ± 0.95 to 80% ± 0.95 for *In vitro* germination.

The physical treatment such as cold, heat, osmotic or nutrient stress apparently triggers a process leading to the expression of desiccation tolerance (Senaratna *et al*., 1990). It can elicit a similar response, presumably because they stimulate the endogenous synthesis of ABA (McKersie *et al*., 1990). Carrot plant produced from a synthetic seed is shown in Fig. 5A, B & C.

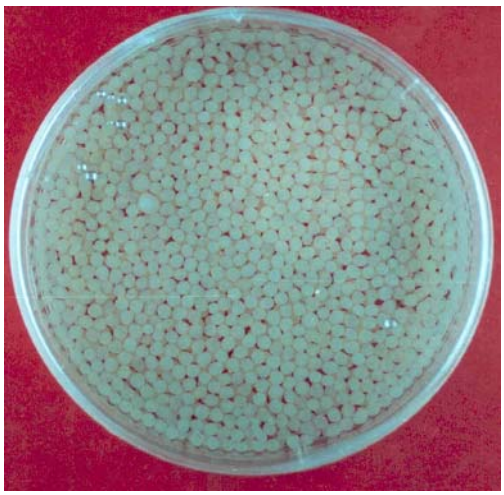

Fig. 3. Somatic embryos encapsulated with Sodium alginate (Synthetic seeds)

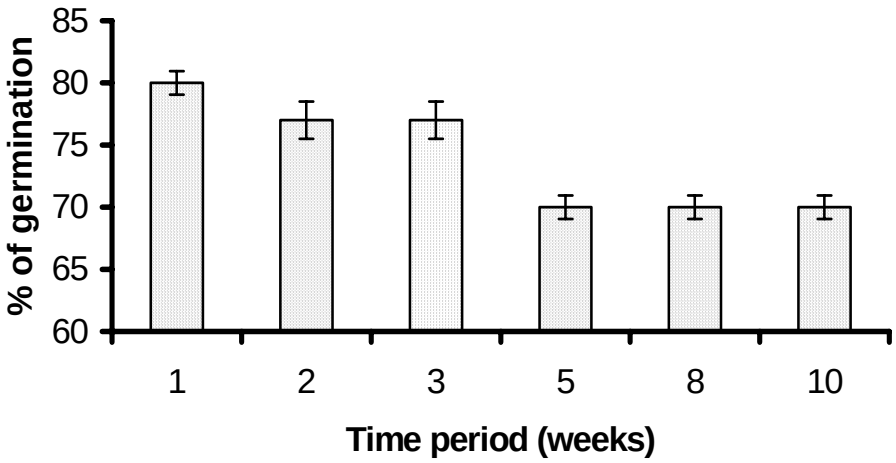

Fig. 4. Germination efficiency of synthetic seeds on MS basal medium.

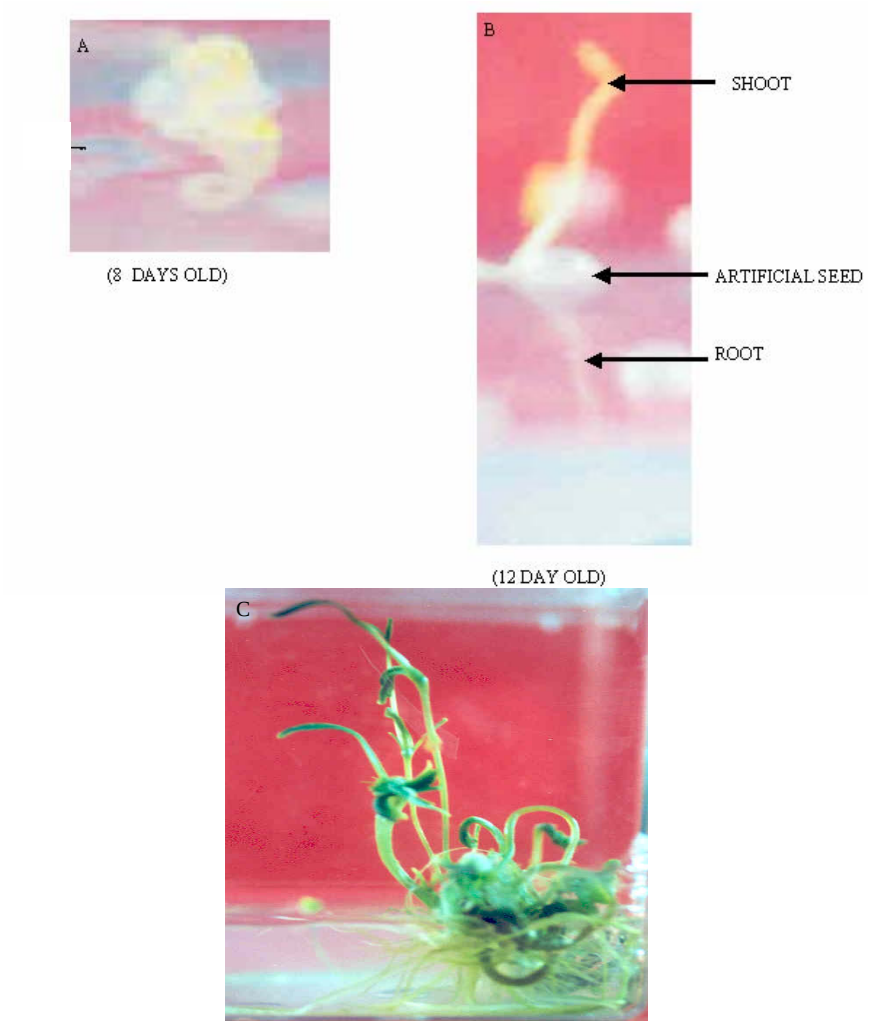

Fig. 5A, B &C. Stages of artificial seed germination.

The main thrust idea of the present work was to introduce a simple inexpensive delivery units of locally produced high yielding crops and a method for direct sowing of encapsulated tissue cultured raised somatic cells called synthetic seeds. Plants can be regenerated on a simplified medium eliminating subcultures, thus regarding the cost of operation. By the development of protocols for direct recovery of plants from synthetic seeds under non-sterile conditions may have a greater impact on the current tissue culture methods, which do not regenerate adequate propagules and are not sufficient to meet the local demand of disease free vegetable seeds. Direct sowing of synthetic seeds in the soil does not need acclimatization often required for the tissue cultured plants. It thus provides an ideal delivery system enabling easy flexibility in handling and transport as compared to large parcels of seedling of plants. The similar system could be developed in

other crops although the specific commercial applications will undoubtedly differ. Synthetic seeds also offer the opportunity to store genetically heterozygous plants or other plant with unique gene combination that cannot be maintained by conventional seed production due to genetic recombination that occurs at each generation of seed increase. The exact application of synthetic seeds will vary from species to species. In cross-pollinating species, especially those where seed production is difficult and expensive, synthetic seeds offer many advantages and opportunities.

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