The effect of activated charcoal on the production and development of somatic embryos in cultures of carrot *Daucus carota*

M Pan and J van Staden*

Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal Pietermaritzburg, Private Bag X01, Scottsville 3209, Republic of South Africa

*Corresponding author, email: vanstadenj@nu.ac.za

Received 16 November 2000, accepted in revised form 6 March 2001

The addition of activated charcoal to Murashige and Skoog medium containing 2,4-D resulted in somatic embryogenesis in cultures of *Daucus carota*. Somatic embryos were not formed in the absence of activated charcoal. In suspension culture, the incorporation of 0.01 to 1.0% activated charcoal to the medium, irrespective of 2,4-D, increased the number of somatic embryos produced. The maximum number of somatic embryos were produced with 1.0% activated charcoal-containing MS medium. Further development of the embryos occurred in the presence of activated charcoal and they regenerated into normal plantlets. Addition of 3.0% activated charcoal to the medium resulted in the formation of abnormal somatic embryos. These embryos produced abnormal plantlets.

Abbreviations: AC – activated charcoal, 2,4-D – 2,4-D Dichlorophenoxy acetic acid, MS – Murashige and Skoog (1962) salts and vitamins

Introduction

The carrot (*Daucus carota* L.) is widely used for studies of somatic embryogenesis (Hanai *et al.* 2000). Morphological and physiological studies have demonstrated that the somatic embryogenesis process consists of at least two phases: firstly, the induction of embryogenic competence in the cells with high concentrations of auxins; secondly, the development of the embryogenic masses into embryos in the absence of or in the presence of, a lowered concentration of auxins (Nomura and Komamine 1985). Somatic embryos develop from embryogenic cells of somatic tissue to form globular proembryos, and progress through the heart-, and torpedo-shaped stages with morphology similar to zygotic counterparts (Michler and Lineberger 1987). Abnormal somatic embryos are known to arise from populations of callus cells (Ammirato 1977). Some of the anomalies described include lateral root development, multiple cotyledons, multiple embryos attached as a single unit, and secondary embryos associated with cotyledons and hypocotyls (Ammirato and Steward 1971). Several factors influence the second phase of the process and the media components, auxin and nitrogen, play crucial roles in manipulating embryogenesis (Köhlenbach 1978). The importance of auxin was first recognised by Halperin and Wehrell (1964). Today, 2,4-D is most commonly used to bring about somatic embryogenesis. The process is often initiated in media containing high levels of auxin. Embryo development is optimised only once the auxin concentration is reduced. After initial induction the development of carrot somatic embryos can generally be enhanced by transferring the embryogenic cells to an auxin-free medium (George 1993).

Activated charcoal is commonly used in plant tissue culture to improve cell growth and development. Activated charcoal may enhance shoot formation and rooting (Fridborg and Eriksson 1975, Patel and Thorpe 1986, Omura and Hidaka 1992, Nour and Thorpe 1993), prevent the development of abnormal plantlets (Ziv and Gadasi 1986) and promote embryogenesis (Fridborg *et al.* 1978, Drew 1979, Johansson 1983, Jones and Petillo 1988, Dethier-Rogers *et al.* 1993). It also restored the embryogenic potential of long-term cultured *Festuca rubra* callus with a declining regeneration potential (Zaghmout and Torello 1988). The effects of AC on *in vitro* culture may be attributed to establishing a dark environment, adsorption of undesirable/inhibitory substances, adsorption of plant growth regulators and other organic compounds, the release of growth promoting substances present in, or adsorbed by, AC or the influence of AC-mediated sucrose hydrolysis during autoclaving (Pan and Van Staden 1998, 1999). A major effect of AC on somatic embryogenesis could well be to adsorb supra-optimal levels of auxin from the culture medium or to influence preferential requirements of explants for the hydrolytic products of sucrose, glucose and fructose. In this investigation the effects of AC on carrot somatic embryogenesis was studied.
Plant material and culture conditions

Seeds of carrot (Daucus carota L. Cape Market) were surface disinfected by immersion in 70% (v/v) ethanol for 2 min and 1.05% (v/v) sodium hypochlorite for 30 min. The seeds were then thoroughly rinsed with sterile distilled water and placed in sterile Petri dishes containing moist filter paper for germination at 25 ± 1°C. When seven-days-old, the seedlings were surface disinfected again with 1.05% sodium hypochlorite solution for 20 min and rinsed five times with sterile distilled water. Ten-mm-long segments were cut from the hypocotyls of the disinfected seedlings and each of the segments placed on medium. Ten replicates were used for each treatment and all experiments were repeated twice.

The media used comprised Murashige and Skoog (1962) salts and vitamins (MS medium). Callus was initiated by placing the hypocotyl sections on MS medium supplemented with 30 g l−1 sucrose, 1.0 mg l−1 2,4-D and 8.0 g l−1 agar. Callus that was produced was subcultured every 3 to 4 weeks on MS medium supplemented with 0.5 mg l−1 2,4-D. Embryogenic suspension cultures formed four weeks after embryogenic callus was suspended in 50 ml Erlenmeyer flasks containing 20 ml liquid MS medium with 0.5 mg l−1 2,4-D. These suspension cultures were kept on a rotatory shaker (100 rpm) and were transferred every 2 to 3 weeks to fresh MS medium with 0.5 mg l−1 2,4-D. The pH of all media was adjusted to 5.8 with KOH or HCl prior to the addition of agar and after the addition of AC. Media were then autoclaved for 20 min at 121°C (118 kPa). All explants and callus were incubated at 25 ± 1°C with a 16:8 light:dark photoperiod under photosynthetically active radiation of 50 μmol m−2 s−1 provided by cool-white fluorescent tubes.

Activated charcoal treatments

Different concentrations of AC (0, 0.01%, 0.05%, 0.1%, 0.5%, 1.0% and 3.0% (v/v)) were added to the MS medium containing 30 g l−1 sucrose with or without 0.5 mg l−1 2,4-D. Solid media were gelled by adding 8.0 g l−1 agar. Callus was cultured on the MS media described above. Suspension cultures (5% v/v) were inoculated into medium with or without 0.5 mg l−1 2,4-D in the presence or absence of AC.

Quantification of somatic embryo formation

Production of somatic embryos was determined microscopically (Olympus BH-2). Somatic embryos progress through four stages of development while undergoing tissue differentiation: globular, heart, torpedo, and cotyledonary stages respectively. The stages are based on the overall embryo shape. In general, carrot somatic embryos remain in the torpedo stage for a few days before progressing to the cotyledonary stage. Cotyledonary-stage embryos have an elongated radicle and hypocotyl, which tends to exhibit hyperhydricity in liquid medium. With suspension cultures 1 ml samples were collected from suspensions every five days and the somatic embryos counted microscopically. Embryos were classified into the various developmental stages according to Shimazu and Kurata (1999). Five replicates were maintained for each treatment and the number of embryos per ml suspension recorded and averaged. The experiment was repeated twice.

Results

With both solid and liquid charcoal-treated media, addition of 0.01 to 3.0% AC induced somatic embryogenesis. After 7 days of culture, globular embryos were observed from callus on all the media used. After being grown for 14 days, a number of globular embryos were observed in the 2,4-D-free medium in the absence of AC (Figure 1A). Heart-shaped and torpedo-shaped embryos developed on all AC-containing media supplemented with 2,4-D (Figures 1B, C, D; 2A, B, C, D, E). Increasing the AC to 3.0% resulted in abnormal embryos, leaf and multiple cotyledons being produced (Figures 1E, F; 2F, G, H). The number of heart-stage embryos increased as the AC concentration used increased up to a level of 0.5% (data not shown). The heart-stage embryos subsequently developed into normal plantlets (Figure 3A). Normal plantlets developed with the low concentrations (0.01 to 1.0%) of AC-treated media. However, abnormal plantlets occurred on the high concentrations (3.0%) of AC-containing media (Figures 3B, C, D). Figure 4 shows the total number of somatic embryos produced per ml of suspension culture when the embryogenic callus was suspended in MS medium supplemented with 0.5 mg l−1 2,4-D in the presence of various concentrations of AC. Within the lower range of AC concentrations used (up to 1.0%) the total number of embryos, which included globular, heart- and torpedo-shape embryos, increased with increasing AC concentration used. However, addition of 3.0% AC to the medium resulted in a decrease in the total number of embryos. Production of somatic embryos also resulted when the callus was cultured in 2,4-D-free MS media in the presence or absence of AC (Figure 4). There were no significant differences in the total number of somatic embryos between 2,4-D-free MS media in the presence or absence of AC (Figure 4).

Figure 5 shows the effect of AC concentration on carrot somatic embryogenesis in terms of the number of somatic embryos in suspension culture with time. The rate of increase in the total number of somatic embryos was affected by the AC concentration, as shown by the similarity of the curves for all treatments. The total number of somatic embryos produced reached a constant level after day 20 for all treatments. The day at which somatic embryos began to appear was not affected by the AC treatment. The number of somatic embryos increased with increased AC concentration when the range of AC concentration was raised upwards from 0.01% to 1.0% in the medium. The number of embryos decreased with the 3.0% AC treatment. From day 20 onwards only half the number of embryos were observed in the suspension treated with 3.0% AC compared to 1.0% AC.
Discussion

The differentiation of carrot cells into somatic embryos occurred when carrot callus was cultured on a 2,4-D-containing medium in the presence of AC, but not on the 2,4-D-containing medium in the absence of AC. This seems to be in agreement with the fact that embryogenesis in carrot cell cultures is inhibited by high levels of auxin (Reinert 1958). It seems that the effects of AC are mainly due to the adsorption of substances such as 2,4-D from the medium. Using chromatography it was confirmed that 2,4-D is effectively adsorbed by AC from aqueous solutions (data not shown). Activated charcoal is known to have the ability to adsorb certain growth regulators that are added to the medium such as NAA, cytokinins (Weatherhead et al. 1978) and ABA (Johansson et al. 1982). The process of carrot somatic embryogenesis is initiated in media containing high levels of 2,4-D, the embryos do not develop until the 2,4-D concentration is reduced. Thus, if the embryos produce one or several hormones that are unnecessary for embryo growth and these substances diffuse into the medium the added AC could
Figure 2: Somatic embryos of Daucus carota after culturing on media with various concentrations of AC showing normal and abnormal embryo development. On 0.01% AC-containing medium, globular embryos were observed but formation of the cotyledons did not occur (A, B, C). On the 0.1% AC-containing medium, cotyledons did develop but the radicles and hypocotyls did not elongate (D). Elongation of the embryos did occur with 0.5% AC in the medium (E). Abnormal embryos with multiple cotyledons developed on the 3.0% AC-containing medium (F, G, H). Bar = 350μm
adsorb and remove these substances from the medium.

The results showed that addition of 0.01 to 1.0% AC increased the total number of carrot somatic embryos in suspension cultures in both 2,4-D-containing and 2,4-D-free media. This indicates that embryogenesis of *Daucus carota* is dependent on the AC concentration used in the medium. AC concentration was an important factor for embryogenesis in *Datura innoxia* and *Solanum tuberosum* (Sopory et al. 1978, Tyagi et al. 1980). The present results are in contrast to those obtained by Wernicke and Kohlenbach (1976) and Johansson and Eriksson (1977) who showed that embryogenesis in *Nicotiana tabacum* and *Anemone virginiana* was independent of AC concentration in the culture medium. However, addition of a high concentration of AC (3.0%) to

---

**Figure 3**: Regenerated *Daucus carota* plantlets from somatic embryos grown in media with different concentrations of AC. A: 0.5% AC treatment, B, C, and D: 3.0% AC treatment.
the medium reduced somatic embryogenesis and induced abnormalities. The somatic embryo response to AC concentration may vary with the plant species, cell density, cell cluster size and cell lines used and it also may be related to the fact that AC was able to adsorb nutrient components from the culture medium. This could result in a nutrient deficiency. The nutrient deficiency could stop further embryogenesis if not compensated for by transfer of the cultures to fresh medium. Many materials are adsorbed or desorbed to colloidal soil particles and AC acts similarly (Proskauer and Berman 1970). Desorption from AC is generally a very slow process depending on solvent and solution conditions.

A number of late-stage embryos appeared in the AC-containing media with or without 2,4-D (data not shown). It seems that AC also provided a major benefit in the progression of later embryo stages in Daucus carota culture. Several factors can influence somatic embryo formation such as cell density and chemical substances which may be secreted into the culture medium from the cells (Hanai et al. 2000). Activated charcoal is able to adsorb all or some 2,4-D added to the medium or metabolites produced by the cells and excreted into the medium. It also adsorbs inhibitory substances such as 5-hydroxymethyl-2-furaldehyde, ethylene and phenolic compounds (Bon et al. 1988, Ebert and Taylor 1990, Fridborg et al. 1978, Horner et al. 1977, Theander and Nelson 1988, Weatherhead et al. 1973, 1979) produced by medium and cultures/embryos, that may inhibit embryogenesis and further embryo development. Addition of an appropriate concentration of AC to the culture medium may result in adsorption of substances produced by the cultures and thus promote somatic embryogenesis.

Acknowledgments — The financial support of the National Research Foundation (NRF), Pretoria, Republic of South Africa is greatly appreciated.

References

Figure 4: Number (average of 10 replicates ± SE) of Daucus carota somatic embryos produced from tissue in MS liquid media with various concentrations of AC in the presence or absence of 2,4-D

Figure 5: Mean number (average of 10 replicates ± SE) of Daucus carota somatic embryos produced over time per unit volume of the AC-containing culture medium
Edited by P Berjak


