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Bioreactor studies of the effect of medium pH on carrot (*Daucus carota* L.) somatic embryogenesis

Véronique Jay*, Simone Genestier & Jean-Claude Courduroux
Laboratoire de Biologie et Physiologie Végétales, Université Blaise Pascal, 4 rue Ledru, 63038 Clermont-Ferrand Cedex 1, France (present address: Greentech, 10/12 avenue Léonard de Vinci, 63063 Clermont-Ferrand Cedex 1)*

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Abstract

Daucus carota cell differentiation was examined under different medium pH conditions in a controlled bioreactor. Somatic embryogenesis was affected by pH changes. Embryo production was greatest when the pH of the hormone-free medium was maintained at 4.3. However, the same level was not favourable to development since most embryos did not progress to the torpedo and plantlet stages. In contrast, although there was about a threefold decrease in embryo yield in cultures on the same free 2,4-dichlorophenoxyacetic acid medium maintained at pH 5.8, cells differentiated into fully developed plantlets. Changes in embryo development appeared to be associated with alterations in ammonium loss from the medium and sugar uptake.

Abbreviation: 2,4-D – 2,4-dichlorophenoxyacetic acid

Introduction

Producing plantlets from suspension culture via somatic embryogenesis may be a useful alternative to traditional propagation systems. Although much work has been done on somatic embryogenesis, there is a lack of basic knowledge concerning the process of plant differentiation. Improvement of production efficiency requires a better understanding of the influence of culture conditions on cell cultures. Bioreactor systems are well suited for this purpose as they are designed to precisely regulate several environmental parameters such as temperature, pH and dissolved oxygen or other gases.

In this work, we investigated the effect of medium pH on carrot somatic embryogenesis. The influence of pH on carrot cell differentiation has already been studied in Erlenmeyer flask cultures by Wetherell & Dougall (1976) who showed that embryogenesis has a rather narrow optimum at pH 5.4. Modifications in growth and development of cells by pH occur mainly by affecting ammonium and nitrate utilization and sucrose uptake (Martin & Rose 1976; Wetherell & Dougall 1976). The role of pH in carrot cell differentiation has also been investigated by Smith & Krikorian (1990 a,b) on a semi-solid medium in which a low pH near 4 sustained the multiplication of pre-globular stage proembryos cultured on a hormone-free medium while preventing development to later stage embryos. A pH buffered medium at 5.8 allowed continuous embryogenic cell differentiation through later stages of embryogenesis.

The aim of this work was to study the effects of pH changes on the differentiation of carrot cells cultured in a bioreactor. The only published report in which a fermentor was used to assess the influence of pH was a study of the somatic embryogenesis of alfalfa (Stuart et al. 1987). We performed two experiments in which the pH of the medium was maintained constant either at 4.3 or 5.8 and determined the effect of culture conditions on the yield and development of embryos. Ammonium loss from the medium and sugar uptake in these cultures were also determined.

Materials and methods

Plant material

The initial cell suspension was established from hypocotyls of domestic carrot (*Daucus carota* L.). It was subcultured in Murashige & Skoog (1962) liquid medium with adenine (11.7 µM) and 2,4-D (1.8 µM). Every ten days, 2 ml of packed cell volume were filtered through steel mesh. Cell clumps which size was between 100 and 500 µm were inoculated into Erlenmeyer flask containing 100 ml of fresh medium. The cell culture was maintained at 26°C on an orbital shaker (100 rpm) under a photoperiod of 16 h. The fluorescent light source (maximum fluence rate 45 µmol m^{-2} s^{-1}) consisted of 58W white daylight tubes (Mazdafluor LJ). The cell suspensions were used as inoculum for bioreactor culture.

Culture conditions

Cultures were grown in a 3 litre bioreactor (Applikon – Holland) containing 1.7 l of fresh medium. Aeration was maintained at a constant rate of 150 ml min^{-1} and the agitation speed was between 50 and 100 rpm depending on the increase in the biomass. The bioreactor was inoculated with 850 mg (fresh weight) of 10 day old aggregate cells between 50 and 100 µm in size. The medium used was the same as that used for Erlenmeyer cultures except that it contained no 2,4-D.

pH regulation

A constant pH level was achieved by a bioprocessor (ADI 1020 – Applikon) that converted the milliamps signals from the pH probe to digital signals which were then recorded on the computer (Tandon PC) with appropriate software (Biowatch – Applikon). From the control factors and the signals from the pH probe, the bioprocessor was able to activate peristaltic pumps to keep the pH at the required level. HCl (1N) and KOH (1N) were used to maintain the media at pH 4.3. The amount of HCl required to maintain the pH at 4.3 was fivefold higher than that of KOH. To sustain the pH at 5.8, KOH (1N) was added to the culture medium.

Assays of the culture medium

Every few days, a sample of medium was filtered on a cellulose acetate filter of 0.2 µm pore size.

For the determination of ammonium concentration, 50 µl of culture medium previously diluted with two volumes of water were added to 3 ml of a mixture containing alkaline hypochlorite (500 µl) and phenol nitroprusside (500 µl) solutions (Sigma, France). The change in absorbance at 570 nm was monitored over a period of 30 min at ambient temperature with a Shimadzu spectrophotometer.

Sucrose, fructose and glucose were assayed with a diagnostic kit (Boehringer, France).

Quantification of somatic embryos

After a culture period of 20 days, about 100 ml of medium were removed and the embryos counted as previously described (Jay et al. 1992).

Results

A characteristic factor associated with cell differentiation was medium pH (Fig. 1). After sterilization and inoculation, the pH was 5.3; it declined during the first 8 days to 4.3, remained constant to day 11 then increased to reach 5.2 at day 20. Since the pH of the medium fluctuated during cul-

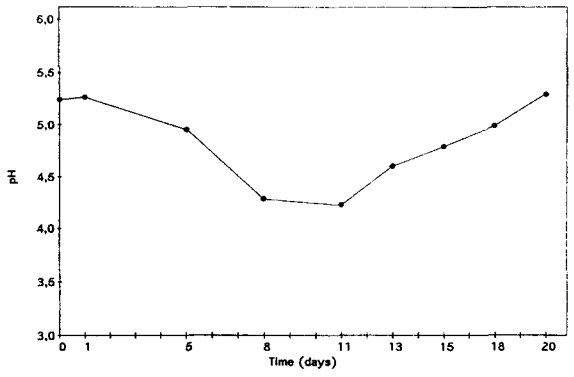

Fig. 1. pH measurement during carrot somatic embryo differentiation into plantlets.

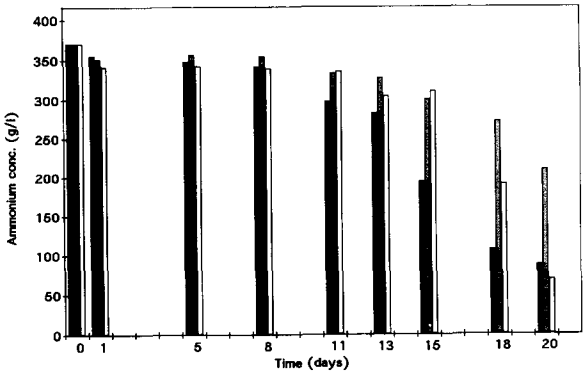

Fig. 2. Effects of pH on ammonium concentration in the medium during carrot cell differentiation. Control culture ■, pH maintained at 4.3▩, pH maintained at 5.8 □.

Table 1. Effects of pH on somatic embryo production

	Control	pH 4.3	pH 5.8
SE/ml	420±130	770±180	135±110

ture, it is likely that the changes in pH affected cell differentiation.

When the medium pH was held constant, embryo production was altered (Table 1). When the pH of the 2,4-D-free medium was 4.3, embryo differentiation was about 45% higher than that obtained in the control bioreactor (without regulation). In contrast, when the pH was sustained at 5.8, embryo production was inhibited threefold.

The effect of pH on embryo development was also investigated (Table 2). The adjustment of pH delayed embryo appearance since the first globular stages were seen at days 8 and 11 at pH 4.3 and 5.8 respectively, whereas they were observed on day 5 in control cultures. Most embryos obtained at pH 4.3 did not progress beyond the heart stage. In contrast, with a pH of 5.8, embryos matured into plantlets which were more developed (larger roots, individualized green cotyledons) than in control culture (data not shown).

The kinetics of ammonium loss from the medium was determined under the three culture conditions (Fig.2). In the control bioreactor culture, NH^{4+} loss began after day 8 and was low between days 8 and 13. Thereafter NH^{4+} content decreased, and by day 20, 80% of the initial ammoium had been removed from the medium. When the pH was maintained at a constant 4.3, ammonium loss was lower than in control since the NH^{4+} concentration slowly decreased and was only reduced by 44% at the end of the cycle. At pH 5.8 we observed a low NH^{4+} loss between days 1 and 15, after which the ammonium content decreased rapidly in the medium. Despite a low embryo production, 80% of NH^{4+} was deleted at day 20, as in control cultures. This result is related to the maturing of embryos into well developed plantlets at pH 5.8.

We also observed changes in the kinetics of sugar uptake during the differentiation phase (Fig.3). From control culture conditions, it was observed that sucrose hydrolysis occurred during days 1 to 15. Glucose and fructose were simultaneously used from day 13. After 20 days of culture, 5 g l^{-1} of hexoses remained in the culture medium. At a pH of 4.3, sucrose hydrolysis was complete on day 13. At the same time, glucose and fructose uptake had begun, but uptake was lower than in control culture, since 13 g l^{-1} of hexoses remained in the culture medium at day 20. Unlike in control cultures, there was preferential use of glucose. At pH 5.8, sucrose hydrolysis occurred throughout the culture period while glucose and fructose were simultaneously taken up from day 15. At day 20, sugar concentration in the medium was 9 g l^{-1}.

Table 2. Effects of pH on embryo development

	Control				pH 4.3				pH 5.8			
	Embryo stages											
Time in days	G	H	T	P	G	H	T	P	G	H	T	P
D_1												
D_5	●	•										
D_8	●	●	•	•	●	●						
D_{11}	●	●	●	●	●	●			●	●	●	●
D_{13}					●	●			●	●	●	●
D_{15}					●	●	•					
D_{18}					●	●	• ⋆	•				
D_{20}					●	●	• ⋆	•				

G:globular stage; H: heart stage; T: torpedo stage; P: plantlet stage.

● : embryo stages in high proportion; • : embryo stages in low proportion compared to all population.

⋆: For other one experiment achieved at 4.3, few plantlet stages were observed whereas for the other one, no plantlets were seen.

Discussion

The pH of the medium can affect carrot cell differentiation. The maintenance of pH at 4.3 enhanced embryo production to the heart shaped stage. Use of this medium may provide an alternative for synchronization of carrot embryogenesis without filtration and centrifugation procedures (Warren & Fowler 1977; Fujimura & Komamine 1979).

The effect of acid pH on carrot cell differentiation was observed by Smith & Krikorian (1990 a, b). They reported that a hormone-free medium at pH near 4 halted embryo development at the globular stage and that serially embryogenic globule subcultures on pH 4 hormone-free medium was able to sustain the multiplication of 2,4-D initiated embryogenic carrot cells. These results could explain why we observed higher embryo production at pH 4.3. It is likely that pH 4.3 both stimulates embryogenic cell proliferation and provides an adequate environment for cell differentiation to heart stage. The preferential use of glucose supports this hypothesis since it has been reported to be characteristic of the proliferation of unorganized carrot cells (Dijkema et al. 1988).

The inhibition of embryo maturity to heart stage by pH 4.3 can be associated with an inhibitory effect on ammonium and sugar uptakes. Martin & Rose (1976) have already shown that the uptake of NH^{4+} and sugar by *Ipomoea* cell suspensions was lower at pH 4.8 than at pH 5.6. Schubert et al. (1990) observed that some ion uptake of field beans decreased at pH 4 and concluded that the sensitivity of field beans to low pH was related to an inability to release protons. In our experimental conditions, media were sustained at pH 4.3 with HCl and KOH. However, since the quantity of HCl required to maintain pH at 4.3 was important compared to that of KOH (five times higher), ATPase driven proton release was probably sufficiently reduced to decrease some nutrient uptake thereby partially inhibiting embryo development.

A higher pH of 5.8 inhibited embryo production. The simultaneous uptake of hexoses which was observed indicates the absence of unorganized cells (Dijkema et al. 1988). Despite the lower embryo production, pH 5.8 allowed embryogenic cells to differentiate to fully developed plantlets. This was associated with high NH^{4+} loss and sugar uptake at the end of the culture.

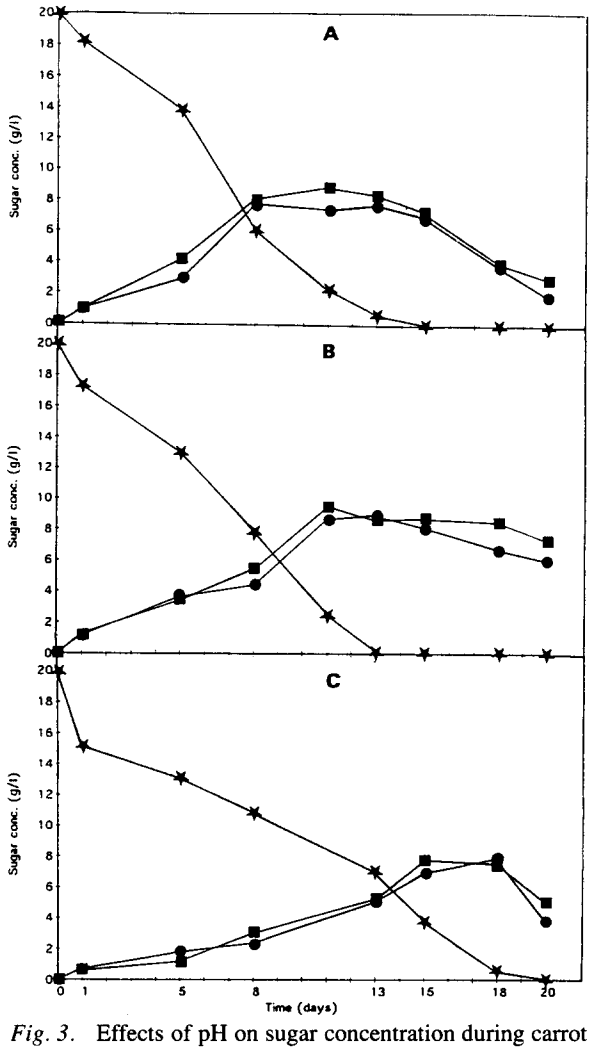

Fig. 3. Effects of pH on sugar concentration during carrot cell differentiation. Sucrose★, glucose ●, fructose ■. (*A*) control culture, (*B*) pH 4.3 culture, (*C*) pH 5.8 culture.

Cell response to pH depends on the stage of differentiation. A low pH (4.3) was best in the first steps of differentiation, whereas a higher pH (5.8) was more favourable to the development of embryos into plantlets. These results suggest that pH could help in optimizing and synchronizing the production of somatic embryogenesis in other species previously considered difficult to synchronize.

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