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Thermochimica Acta 447 (2006) 52–56
thermochimica acta
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Metabolic heat and CO_2 evolution rates measured by calorimetry during different in vitro induction processes of cineraria

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Received 2 January 2006; received in revised form 4 April 2006; accepted 20 April 2006
Available online 7 May 2006

Abstract

Explanted cotyledons from cineraria 'Jester Pink' seeds were cultured in vitro on a modified Murashige–Skoog medium containing (in μM): 13.5 NAA, 13.5 2,4-D, 4.4 BAP, 13.5 NAA + 4.4 BAP, 13.5 2,4-D + 4.4 BAP or no plant growth regulators (control). Photographs were taken at 7-day intervals for 35 days to follow the embryogenic/organogenic development of the explanted cotyledons. Calorespirometric (metabolic heat rate, R_q and respiration rate, R_{CO_2}) measurements were made in triplicate on the cultured cotyledons at the same interval as the photographs. Results showed a close correlation between the embryogenic/organogenic response of the tissues and R_q values. Cotyledons on a medium containing 2,4-D + BAP had the highest R_q values while those cotyledons that failed to develop or those of control, that suffered deterioration, had the lowest. This work showed that calorespirometric data may be useful in estimating early responses of tissues/organs in vitro embryogenic/organogenic culture systems.

Keywords: CO_2 production; Respiration; Micropropagation; Somatic embryogenesis; Organogenesis; Plant growth regulator

1. Introduction

An embryo, a miniature plant bearing both shoot and root apices, can arise from gametic fusion (zygotes) following sexual reproduction (zygotic embryos) or can be induced asexually (somatic or nonzygotic embryos [3]). It is the clearest manifestation of totipotency, the potential to regenerate entire organisms, that, theoretically, all living plant cells possess [6].

Somatic embryogenesis has become a useful tool for plant micropropagation, artificial seed technology, somaclonal variation, and genetic transformation of agronomic, forestry and horticultural, especially ornamental, species. Although somatic embryogenesis has been described for more than a hundred plant species from different families, the number of reports on members of the *Asteraceae* is still limited. Despite the extensive research on somatic embryogenesis, our understanding of this process is far from complete. Low conversion rate of embryos into intact plants has been a serious problem for this technique to be of practical use [12].

Cineraria (*Asteraceae*) is a colorful potted crop that is commercially propagated by seeds which are heterozygous for flower color [8]. Somatic embryogenesis in cineraria may be used as a tool for clonal propagation eliminating the problem of propagule segregation. Somatic embryogenesis in ornamental plants can be more efficient than conventional vegetative propagation and other micropropagation techniques such as axillary shoot multiplication and organogenesis [10].

Plantlet production via the process of somatic embryogenesis is now firmly established as an important tool for plant propagation. However, a better theoretical understanding of the process is still necessary.

The use of calorimetry may provide a method to study how explanted tissues and/or organs respond to differing tissue culture conditions. Calorimeters measure metabolic parameters of respiring plant tissues, such as the rate of metabolic heat production (R_q) and rates of CO_2 emission (R_{CO_2}) under various environmental conditions. With this technique, it may be possible to rapidly predict developmental responses of tissues and/or organs cultured in different environments based on metabolic parameters. Calorimetry has a distinct advantage over traditional methods due to its rapidity of measurement and reduction of maintenance costs of poor performing plants in culture.

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0040-6031/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.tca.2006.04.015

To date, there has been little calorespirometric research focused on plant tissue culture with the majority of work involving fungal or animal cultures. Research by Fontana et al. [4] has shown that cell cultures of tomato in liquid medium can be studied calorimetrically. To increase oxygen availability/diffusion and avoid cell desiccation, they floated tomato cells on a high density Percoll solution within the ampoule. This eliminated the metabolic limiting factor of oxygen diffusion and dramatically increased metabolic rates compared to cultures without Percoll. Plant callus tissues on gelled agar have also been tested calorimetrically with good results [1].

This study was conducted to investigate the conditions involved in efficient somatic embryogenesis and plant regeneration of cineraria 'Jester Pink' and to determine whether calorespirometric measurements could be used to follow embryogenic or organogenic responses.

2. Materials and methods

2.1. Explant preparation

Approximately 24 seeds per 15 cm diameter disposable Petri dish, totaling about 600 cineraria *Senecio cruentus* 'Jester Pink' seeds (Ball Horticultural Company, West Chicago, IL, USA) were sown on June 21, 2004 on an MS medium in 25 petri dishes each with 25 mL MS basal medium. Seeds were germinated in the culture room with 16 h photoperiod of 40 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) provided by cool-white fluorescent lamps, at 25 ± 1 °C. For disinfection, seeds were immersed and vortexed for 1 min first in 70% (v/v) ETOH, and then in 1% (v/v) sodium hypochlorite solution. Seeds were rinsed three times in sterilized double-distilled water. Cotyledons of uniform size and color were harvested as explants from the 9-day-old germinated seedlings. Each explant was cultured on a modified MS medium [9] supplemented with plant growth regulators (PGR).

2.2. Preparation of the treatment media

The MS basal medium was prepared with premixed MS salts [9] (Gibco Laboratories, Life Technologies, Inc., Grand Island, NY, USA), B_5 Vitamins [5], 3% (w/v) sucrose (EMD Chemicals Inc., Gibbstown, NJ, USA), and 1.5% (w/v) Bacto agar (Becton Dickinson and Company, Sparks, MD, USA). The PGRs used were 13.5 μM 2,4-D (2,4-dichlorophenoxyacetic acid; Sigma Chemical Co., St. Louis, MO, USA) or 13.5 μM NAA (α-naphthaleneacetic acid; Sigma Chemical Co., St. Louis, MO, USA) and 4.4 μM BAP (benzyladenopurine; Sigma Chemical Co., St. Louis, MO, USA), alone or in combinations (Table 1). The pH of medium was adjusted to 5.8 using 0.1N NaOH or HCl before autoclaving.

Each 400 mL treatment medium was first microwaved for 5 min to dissolve ingredients and 5 mL was dispensed into scintillation culture vials (Liquid Scintillation Vial, Wheaton Scientific, Millville, NJ, USA) before autoclaving at 121 °C for 15 min. Each cotyledon was cut on the petiole and distal sides. Cotyledon explants (2–3 mm × 3–4 mm) were planted with the adaxial side up on the induction medium.

Table 1
Compositions and concentrations of 2,4-D, NAA, and BAP supplemented to the MS medium for the induction of different developmental processes in cotyledons of cineraria 'Jester Pink'

Treatment no.	Plant growth regulators (μM)		
	2,4-D	NAA	BAP
1 (control)	0.0	0.0	0.0
2	0.0	13.5	0.0
3	13.5	0.0	0.0
4	0.0	0.0	4.4
5	0.0	13.5	4.4
6	13.5	0.0	4.4

In each treatment 80-culture vials with 5 mL treatment medium in each was used. Culture vessels in each treatment were put in a cardboard holder. Explants were first incubated for 2 weeks in the dark by covering with aluminum foil. They were then kept under a 16 h photoperiod of 40 μmol m^{-2} s^{-1} photosynthetic photon flux provided by cool-white fluorescent lamps, at 25 ± 1 °C.

2.3. Microscopic observations

The developmental or deterioration process in each treatment was observed with a stereo microscope (Stemi 2000-C, Zeiss, Germany), at weekly intervals and digital images were taken with a digital still camera (DSC-F717, Sony Corp., Japan). At the same time, metabolic heat and CO_2 evolution rates were measured.

2.4. Calorespirometric measurements

Calorespirometric measurements were made 0, 1, 2, 3, 4 and 5 weeks after culture initiation with a CSC (Calorimetry Sciences Corporation, Provo, UT) Model 4100 differential scanning calorimeter operated in isothermal mode (20 °C) with the water bath temperature of 15 °C. The calorimeter had four removable ampoules, three of which were used for simultaneous measurements of rate of heat production (R_q) with the remaining ampoule used as a reference. Ampoules were thin-walled cylinders of 1 cm^3 volume, constructed of Hastelloy C, with a screw cap sealed with a Viton gasket. The rate of CO_2 production (R_{CO_2}) was determined via the methods of Criddle et al. [2] and Fontana et al. [4]. R_{CO_2} measurements were facilitated by using the bottom 3 mm of a thin-walled micro Eppendorf tube. Called a 'base trap', these small containers were filled with 40 μL of 0.4N NaOH and inserted into the ampoules to remove CO_2 produced by respiring tissues. The heat of the exothermic reaction between CO_2 and NaOH forming carbonate was used to calorespirometrically estimate respiration rates. The CO_2 reaction heat rate divided by the change in enthalpy between the reaction of CO_2 with NaOH in the base trap (108.5 μJ nanomol^{-1}) was used to determine the rate of CO_2 production (R_{CO_2}) [7].

Three measurements were taken for each treatment each week and number of tissue samples put in each ampoule for one measurement were 11, 8–9, 5, 3, 3, 2–3, and 1–3 for weeks 0, 1, 2, 3,

4, and 5, respectively. The number of samples needed each week for calorespirometric measurements decreased as the tissues got larger. Tissues were taken out of culture vessels and any remaining culture medium was blotted off by placing them on paper tissue (Kimwipes EX-L, Kimberly-Clark Corp., Rosewell, GA, USA) before placing them in measurement ampoules. Samples of each treatment were put in all three ampoules at three different measurements in rotation in an effort to reduce the artifact arising from location in the calorimeter. After calorimetric measurements, tissue samples were measured for fresh and dry mass. Tissue dry mass was determined after overnight drying in an 80 °C vacuum (15 in.Hg) oven (Model 5831, Napco, Precision Co, VA, USA) and dry mass data were used in the calculations of R_q and R_{CO_2} values.

2.5. Statistical analysis

Data obtained were analyzed for statistical significance by using the SAS program (Statistical Analysis System, V. 6.12, Cary, NC, USA). Treatment means were compared using standard deviations.

3. Results and discussion

Photographic analyses and calorespirometric (metabolic heat rate, R_q and respiration rate, R_{CO_2}) measurements of the explants showed a striking parallelism. In general, when explants failed to develop their R_q and R_{CO_2} values were low, when they showed some development short of embryogenesis their R_q and R_{CO_2} values were intermediate and when they formed somatic embryos their R_q and R_{CO_2} values were highest. Differences among treatments were clearly apparent in just 14 days of culture.

3.1. Metabolic heat rate (R_q)

Initially, during the first 7 days, R_q values for all treatments increased (Fig. 1). This kind of response has been observed before with recently explanted tissues in vitro [11]. They characterized an initial increase in R_q values as a wound response which could apply here as well. Support for a wound response rise in R_q came from the photographic analysis (Fig. 3). None of the explanted cotyledons showed any embryogenic or organogenic development in the first 7 days. At day 14 and thereafter, the R_q value for the control treatment (devoid of any growth substances in the culture medium) decreased rapidly and steadily. Photographs of explants in the control treatment showed no tissue development and eventual necrosis of the tissue. In comparison, R_q values for explants on the BAP treatment kept constant for 28 days, and decreased to reach by day 35, a significantly higher R_q value than the control, but lower than for any of the other treatments. This calorimetric result was also corroborated by the photographic analysis; explants on BAP alone showed no organogenic or embryogenic development. Explants on 2,4-D, NAA + BAP and NAA had stable R_q values throughout and by day 35 had similar R_q values that were higher than the control and BAP treatments, but lower than the 2,4-D + BAP treatment. The photographic analysis showed that explants on 2,4-D alone formed embryos, but they were soft and stopped development at the globular stage (Fig. 3(4-D)). Explants on NAA alone formed adventitious roots (Fig. 3(5-B)) and explants on NAA + BAP eventually formed shoots (Fig. 3(5-E)). The 2,4-D + BAP treatment had the highest R_q values on day 14 and at the end of the experiment on day 35. Explants on this medium also had the highest embryogenic activity eventually forming bright yellow somatic embryos (Fig. 3(5-F)). Only explants that formed roots on a medium containing NAA had R_q values that were similar (Fig. 1).

3.2. Respiration rate (R_{CO_2})

In general, there were few major differences in R_{CO_2} amongst any of the treatments from day 0 to day 28 (Fig. 2). However, on day 35 the 2,4-D + BAP treatment having the greatest embryogenic activity had the highest R_{CO_2} value while the control and BAP treatments had the lowest. There was

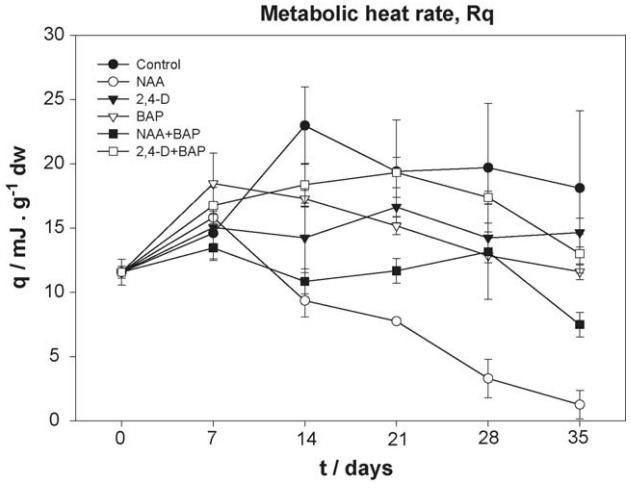

Fig. 1. Metabolic heat rate, R_q, of explanted cineraria cotyledons on different PGR media during 35 days.

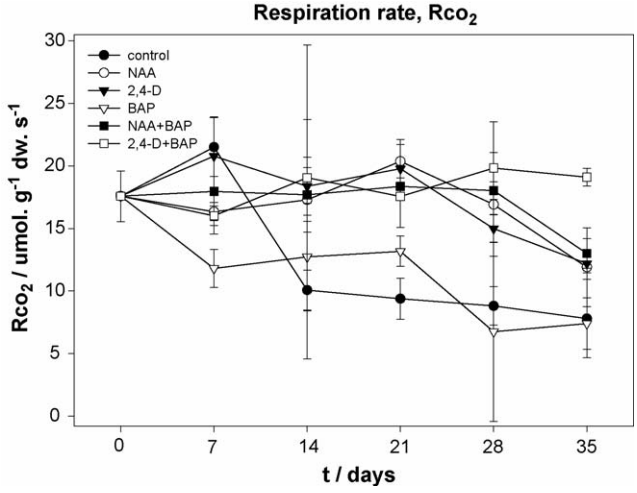

Fig. 2. Respiration rate, R_{CO_2}, of explanted cineraria cotyledons on different PGR media during 35 days.

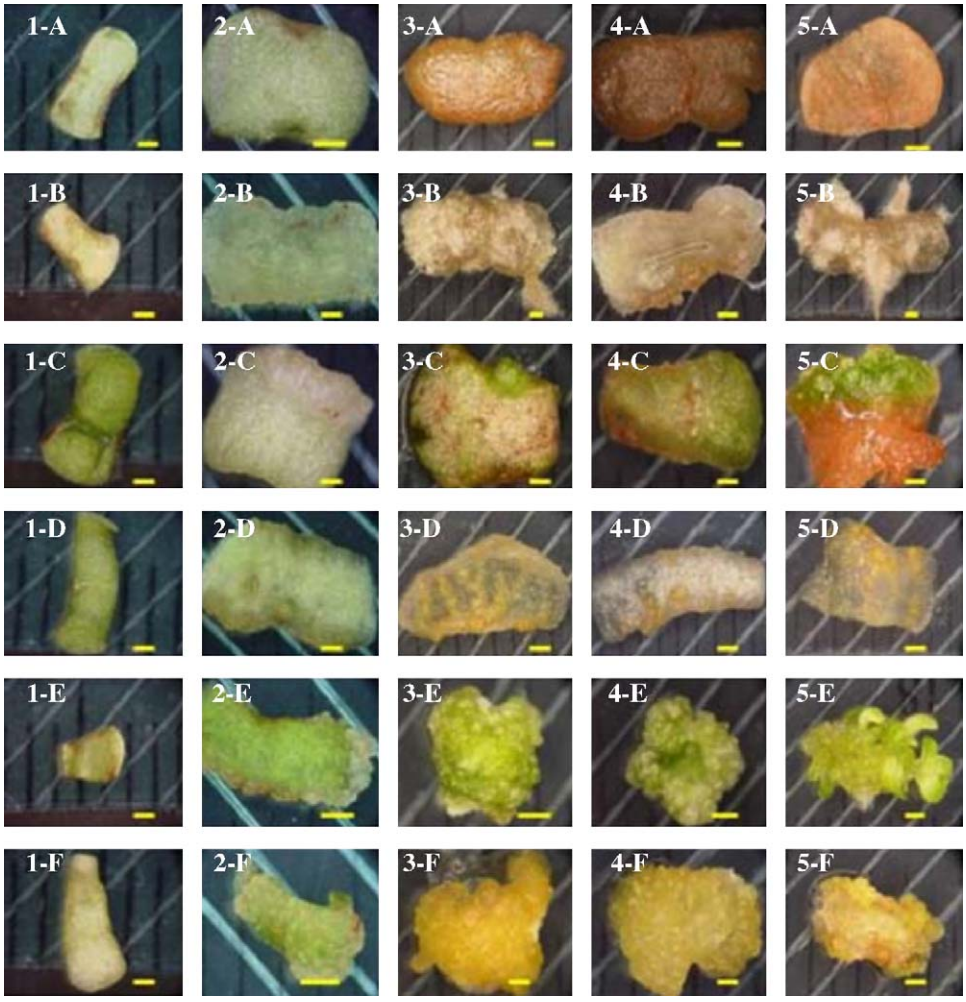

Fig. 3. Ontogenic development of explanted cineraria cotyledons on different PGR media after: (1) 7; (2) 14; (3) 21; (4) 28 and (5) 35 days in: (A) control (without PGR); (B) 13.5 μM NAA; (C) 4.4 μM BAP; (D) 13.5 μM 2,4-D; (E) 13.5 μM NAA + 4.4 μM BAP and (F) 13.5 μM 2,4-D and 4.4 μM BAP. ((yellow) bars = 10 mm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

significantly more variation in the R_{CO_2} data so additional treatment differences were not found.

4. Conclusions

Cineraria cotyledon explants responded in classic ways to plant growth regulators in culture media. On a medium without growth regulators the cotyledons failed to develop; on a medium with NAA they formed roots; on a medium with BAP + NAA they formed shoots and on media with 2,4-D with or without BAP they formed somatic embryos. The calorespirometric responses that paralleled the morphogenetic responses showed that calorimetry was able to detect differences in developmental response in just over 14 days and, therefore, may be able to play a predictive role assessing plant tissue culture experiments aimed at eliciting a morphogenetic response. In this case, the calorespirometric results showed in an early stage (14 days) of the experiment that the explants of the control treatment were deteriorating (lowest R_q values) and that those of the 2,4-D + BAP, that at the end formed somatic embryos, had the highest metabolic activity. This ability to indicate or predict morphogenetic responses may be of significant help in assessing tissue culture media in other systems.

Acknowledgement

This study was financially supported by the Brain Korea 21 project.

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