Scientia Horticulturae 127 (2011) 542–547

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Micropropagation of dahlia in static liquid medium using slow-release tools of medium ingredients

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ARTICLE INFO

Article history:
Received 16 August 2010
Received in revised form
10 November 2010
Accepted 17 November 2010

Keywords:
Nutrients
Tissue culture
Dahlia
Refractometer
EC-meter
Osmocote

ABSTRACT

Growth of dahlia shoots in vitro was *ca.* 4 times faster in liquid medium than on solidified medium. In liquid standard medium (3% sucrose, macroelements according to Driver–Kuniyuki Walnut medium, microelements according to Murashige–Skoog medium, 0.44 μM benzylaminopurine), the major medium ingredients were consumed for 75–80% during the first 6 weeks. Addition of extra ingredients increased growth, demonstrating that the amount of ingredients added at the start of culture was suboptimal. When the extra ingredients were given at the start of the culture, concentrations became too high and therefore inhibitory. When the ingredients were added during the subculture cycle by means of small aliquots of a concentrated solution or by means of slow-release tools, growth was strongly increased. Osmocote gave satisfactory results as a slow-release tool for inorganics. For organic ingredients (sucrose and benzylaminopurine), a novel slow-release tool was developed.

1. Introduction

The main ingredients of plant tissue culture media include sucrose, inorganic nutrients, plant growth regulators and vitamins. Their consumption during a subculture cycle has been examined in liquid medium with cell suspensions and organ cultures (Schmitz and Lorz, 1990; Desamero et al., 1993; Holme, 1998; Morard et al., 1999; Lian et al., 2002), and on solid medium with organ cultures (Leifert et al., 1995; Ruzic et al., 2001; Ramage and Williams, 2003). In liquid medium many of the nutrients are being used up. Thus, addition of extra nutrients is expected to bring about more growth. Because an increase of the initial doses may result in concentrations that are unfavorable for growth, addition during the subculture cycle is desirable. This can be done by adding extra nutrients in a small volume of liquid medium with a high concentration (Maene and Debergh, 1985; Aitken-Christie and Jones, 1987). This method, though, is inconvenient in a commercial lab, among others because of the risk of contamination. In the present research we use slow-release tools for inorganic (minerals) and organic (carbohydrates and hormones) medium ingredients. Because of the techniques involved in producing these tools, inorganics and organics cannot be combined in one single device in large-scale production (L. Wagenaar, Katwijk, The Netherlands, pers. comm.).

In horticulture, granules enabling slow release of inorganic fertilizers are being used widely to reduce leaching losses (Maynard and Lorenz, 1979; Sharma, 1979). These granules may be usable in tissue culture. For organics no slow-release tools are available. Slow release may be achieved chemically. Carbohydrates may be added, e.g., as starch which can be slowly broken down into small, usable units. Similarly, plant growth regulators may be added as compounds in which the active regulator is conjugated with another compound (Tsatsakis et al., 1995). In both cases, release is achieved by enzymes but control is difficult for various reasons, among others instability of the enzymes and pH dependence of their action. About the latter it should be noted that pH changes drastically during tissue culture and is uncontrolled (De Klerk et al., 2008). Alternatively, slow release may be achieved physically by encapsulation within membranes that are little permeable or degrade slowly. Wybraniec et al. (2002) studied the release of auxin and paclobutrazol encapsulated in granules together with inorganics. We used another slow-release tool, porous tablets containing sucrose that is released by diffusion. Benzylaminopurine can also be included in this slow-release tool. In our experiments, slow release of vitamins was not considered and the plantlets received only the initial dose of vitamins. In this paper, we report that growth in tissue culture can be extended using the granule-type release for inorganic nutrients and porous tablets for slow release of organics.

Abbreviations: MS, Murashige–Skoog; BAP, 6-benzylaminopurine; DKW, Driver–Kuniyuki Walnut; OSRTs, organic slow-release tools; LM, liquid medium; SM, solidified medium.

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0304-4238/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.scienta.2010.11.015

2. Materials and methods

2.1. Plant material and culture conditions

Established cultures of dahlia (*Dahlia hybrida*) cultivar 611025 were a generous gift of SBW International BV, Roelofarendsveen, The Netherlands. To maintain the stock, 5 explants were cultured in 50 ml static liquid medium at 50 μE m^{-2} s^{-1} (16 h per day) and 20 °C in plastic containers (Wavin) with a diameter of 9 cm. The standard medium contained DKW-macronutrients according to Driver and Kuniyuki (1984), MS-micronutrients according to Murashige and Skoog (1962), 3% sucrose, 0.44 μM BAP. Subculture was every 4 weeks. The explants were either single nodes (a node with *ca.* 1 cm stem and two leaves) or apical sections (*ca.* 1 cm) of shoots.

For the experiments, five single nodes were cultured in 50 ml liquid medium in a Wavin container. Per treatment, three containers were used. So in total, per treatment 15 explants were studied. Extra medium components were added as indicated.

2.2. Measurements of sucrose, total amount of inorganics, individual elements, radioactive BAP and dry weight

The concentration of the total of minerals was estimated with an EC-meter (315i, WTW Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Individual minerals were determined by the service laboratory of the Chemical Biological Laboratory for WUR – Soil Centre (Wageningen, The Netherlands) using SFA-$CaCl_2$ (NH_4^+ and NO_3^-) and ICP-AES (the other minerals).

Sucrose, and its hydrolysis products glucose and fructose were determined enzymatically using an enzyme kit (Boehringer, Mannheim, Germany; sucrose is hydrolyzed by invertase to fructose and glucose; these sugars are measured). The total amount of carbohydrates was measured chemically with anthrone (De Bruyn et al., 1968) and with a digital refractometer (PAL-1; Atago, Tokyo, Japan).

6-Benzylamino[8-^{14}C]purine (2.02 GBq $mmol^{-1}$) was from Sigma (St. Louis, USA). Aliquots (200 μl) of the medium were taken at the indicated intervals and to determine the amount of radioactivity, 4.5 ml Aqua Gold (Packard) was added.

For dry weight determination, the plant material was dried at 70 °C for 3 days.

2.3. Slow-release tools

Osmocote Pro 3-4M was a generous gift of Scott International (Geldermalsen, The Netherlands). The characteristics are shown in Section 3.

The slow release castings for sucrose and BAP were developed by Kiwi Farm (Katwijk, The Netherlands). Each weighed 5 g and contained 60, 40 or 20% (w/w) sucrose and each 0, 0.17, 0.56 or 1.11 μmol BAP. The characteristics are shown in Section 3.

2.4. Statistics

The values of DW, and numbers of nodes are means from 15 explants ± SE. When EC and brix values of culture media were determined, the values are means from three containers ± SE. All experiments were repeated at least twice. In the other chemical determinations (Table 1, Figs. 1 and 4), the values represent single determinations. In this case, the experiment was repeated at least three times. The error bars in the figures are SEs. When no bar is shown, SE is smaller than the symbol. Values having same letter do not differ significantly at 0.05 level. The significances of differences were evaluated by a Student-*t* test.

Table 1
Composition of the inorganics released from Osmocote.

	Osmocote (mg/l)	DKW (mg/l)	DKW/Osm
P	128	49	0.4
NO_3^-	612	387	0.6
NH_4^+	512	200	0.4
S	102	309	3.0
K	591	624	1.1
Na	24	59	2.5
Mg	23	58	2.5
Ca	5.9	299	50.7
Mn	0.7	9	13.0
Fe	0.7	5	7.2

Released inorganics from Osmocote granules (2.5 g) in 500 ml water during 8 weeks. For comparison, the composition of DKW at the same concentration is shown.

3. Results

3.1. Validation of analytical techniques for carbohydrates

For estimation of the total amounts of sucrose and inorganics, quick methods are available, namely, determinations with a refractometer (brix-meter) and with an EC-meter, respectively. Both methods were validated. We compared measurements of sucrose by a refractometer with an enzymatic test and the anthrone determination. We used medium with explants after increasing periods of culture. A determination with the refractometer took only a few seconds. The three determinations gave very similar results (Fig. 1).

An EC-meter measures the electrical conductivity of a solution. The measured value depends on the dissociation of salts in anions and cations. At high concentration, the conductivity is therefore less. At full concentration DKW, the value determined by the EC-meter was 10% less than expected on base of 1/2 concentration DKW (data not shown).

3.2. Growth in liquid and solid media and depletion of the medium

The increase of DW during a 6-week subculture cycle in liquid medium was *ca.* 4 times higher than on solid medium (Figs. 2 and 3A). In the liquid medium, inorganic compounds and carbohydrates were largely exhausted after 6 weeks (Fig. 3B). On solid medium, growth slowed down after 6 weeks and stopped fully

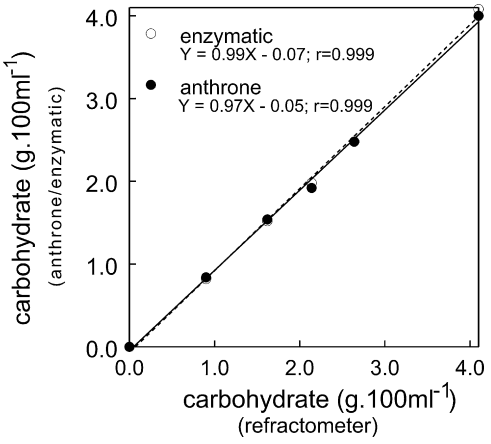

Fig. 1. Validation of carbohydrate determinations by a refractometer. Dahlia shoots were grown in liquid medium. After 0, 2, 3, 4 and 6 weeks samples were taken and carbohydrates were measured by a refractometer, by the anthrone determination (total carbohydrate) and by an enzymatic determination (sucrose, fructose and glucose). The values obtained by the anthrone and enzymatic determinations were plotted as a function of the reading obtained with the refractometer. Note that in this experiment the initial sucrose concentration was 4 g 100 ml^{-1}.