Note

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An Envelope-Shaped Film Culture Vessel for Shikonin Production by *Lithospermum erythrorhizon* Hairy Root Cultures

Hiroshi FUKUI*†, A.F.M. Feroj HASAN*, Yukiko ISHII** and Michio TANAKA**

*Department of Biochemistry and Food Science,
**Department of Horticulture, Faculty of Agriculture, Kagawa University, Kagawa 761-0795, Japan

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Abstract

Hairy roots of *Lithospermum erythrorhizon* cultured in "Culture Bag" containing NH_4NO_3-depleted MS medium without agitation were found to produce shikonin. Although the amount in the Culture Bag was less than that in shaken flasks, the ratio of shikonin secreted into the medium in the former was higher than that in the latter, suggesting that the "Culture Bag" is useful for studies of the physiological effect of shaking or agitation on plant tissue and cell cultures in a liquid medium.

Plant tissue and cell culture in a liquid medium needs agitation or forced aeration to supply oxygen for proliferation. Various types of culture vessels have been devised for supporting the growth; for example, conical and flat-bottom round flasks placed on reciprocal or gyratory shakers, bubble-type reactors (Tulecke and Nickell, 1959), stirred-jar fermentors (Byrne and Koch, 1962; Koge *et al.*, 1992), flat-bladed impeller bioreactors (Hooker *et al.*, 1990), roller-bottle systems (Lamport, 1964; Shibasaki *et al.*, 1992), air-lift column bioreactors (Wagner and Vogelmann 1977; Smart and Fowler 1984), cell-lift impeller bioreactors (Treat *et al.* 1989; Kim *et al.*, 1991) and helical-ribbon impeller systems (Jolicoeur *et al.*, 1992). In these systems, the cultured cells are incapable of surviving without shaking for sufficient oxygen supply. Tanaka (1991) reported that envelope-shaped (named "Culture Bag") and box-shaped (named "Culture Pack") culture vessels made of fluorocarbon polymer film are convenient for micropropagation of ornamental plants. Previously, we (Fukui and Tanaka, 1995) also reported the high production of shikonin by *Lithospermum erythrorhizon* cell cultures with less formation of abnormal metabolites (echinofuran derivatives) (Fukui *et al.*, 1984) in this culture vessel system compared with agitated flasks. The Culture Bag was also found to be suitable for the production of anthocyanin by *Euphorbia milli* cells (Hamade *et al.*, 1994) and artemisinin production in *Artemisia annua* cells (Teo *et al.*, 1995) cultured in a liquid medium. Recently, the hairy roots of various plant species have been reported to be useful for metabolite production as shown by Shimomura *et al.* (1991), Tanaka *et al.*, (1996) and Aoki *et al.*, (1997). This paper reports the productivity of shikonin by the hairy roots of *L. erythrorhizon* cultured in the film culture vessel without agitation.

The Culture Bag was prepared as follows. Sheets of fluorocarbon polymer film (Neoflon® PFA, Daikin Industries, Ltd., Osaka, Japan; 12.5, 25, and 50μm thick, 15 cm long by 20 cm wide) were shaped into an envelope (see **Fig. 1**, for further details refer to Tanaka, 1991). The upper edge of the envelope was cut on a slant. After autoclaving the Culture Bag, the opening which was covered with aluminum foil was used to introduce autoclaved medium (30 m*l*). The four types of media used were Murashige-Skoog (MS) medium (Murashige and Skoog, 1962), ammonium nitrate-depleted MS (MS-NH_4NO_3), MS containing half-strength ammonium nitrate (MS-1/2NH_4NO_3) and M9 medium (Fujita *et al.*, 1981). *L. erythrorhizon* hairy roots (200 mg fr. wt, induced by *Agrobacterium rhizogenes* strain 15834) (Fukui *et al.*, 1998) that had been grown in MS-NH_4NO_3 medium with no hormones and 3% sucrose were inoculated into the Culture Bag containing the medium. After pushing out the air trapped in the upper space in the Culture Bag, the opening was temporarily sealed with a closing device (Spectrum Medical Industries, USA), before heat-sealing. The heat-sealed Culture Bags were laid

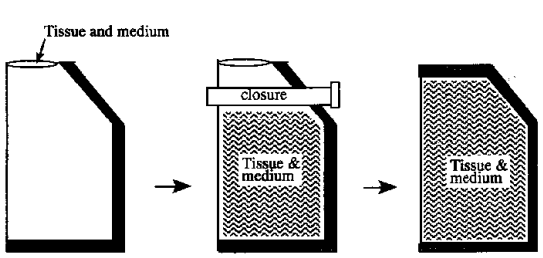

Fig. 1 Diagram illustrating the procedure to make and use the Culture Bag.
The shadowed sides were heat-sealed.

† To whom correspondence should be addressed.

on a one-cm mesh net sheet without overlapping to make the air-permeable film area as broad as possible, and then the nets were piled on top of one another with one-cm or more space between the nets and incubated at 25°C in the dark without shaking for 3 weeks. Control cultures were carried out in 100 m*l* flasks containing the same volume of the same medium as the Culture Bag and agitated on a rotatory shaker (70 rpm) at 25°C in the dark for the same period as the Culture Bag. The Culture Bags and control flasks were taken out twice a day to observe the condition of the tissues inside.

The hairy root cultures were filtered through Miracloth. The hairy roots harvested were freeze-dried, weighed and extracted with ethanol (10 m*l*). The ethanol extracts were adjusted to 20 m*l*, and the filtered medium (*ca.* 30 m*l*) was diluted with ethanol to 60 m*l*. Then an aliquot of the solution was subjected to qualitative and quantitative analyses by HPLC according to our method (Fukui and Tanaka, 1995) . The quantities of shikonin derivatives were estimated as shikonin from the corresponding HPLC peak areas.

The hairy roots of *L. erythrorhizon* proliferated in the Culture Bag containing MS-NH_4NO_3 medium without continuous agitation (**Fig. 2**, standard deviations: 4~20%), although the growth was almost half of that in shaken flasks. The hairy root growth was strongly inhibited by the presence of ammonium ion in all the culture vessels including the shaken flasks. The hairy roots grew more vigorously in the shaken flasks than in the Culture Bag containing MS-NH_4NO_3 medium, probably due to the lack of a sufficient oxygen supply. The hairy root growth in the media containing ammonium ion was the same in all the culture vessels including shaken flasks. These data suggest that the hairy roots need sufficient oxygen without ammonium ion for good growth.

L. erythrorhizon hairy roots produced shikonin derivatives in the Culture Bag containing MS-NH_4NO_3 medium (**Fig. 3**). The amount of shikonin in shaken flasks was larger than that in the Culture Bags. However, the shikonin amount in the Culture Bag containing the other media was similar to that in the shaken flasks. Shikonin production in the Culture Bag containing MS medium was strongly inhibited due probably to the presence of the ammonium ion as reported by Shimomura *et al.*, (1991). M-9 medium that contains no ammonium ion was devised for effective shikonin production by the dedifferentiated cells (Fujita *et al.*, 1981). This production medium was not suitable for shikonin production by the hairy roots used in this study due to their undernourishment. *L. erythrorhizon* hairy roots showed a good relationship between shikonin production and proliferation (**Fig. 2** and **3**) in contrast to the dedifferentiated cell cultures. These results suggest that shikonin production in hairy roots has a direct connection to primary metabolism, while *L. erythrorhizon* cell cultures that accumulate the biosynthetic intermediates such as *p*-hydroxybenzoic acid glucoside (Yazaki *et al.*, 1986) in the MS medium (growth medium of two-stage culture) were transferred to the M9 medium (production medium) to produce a large amount of shikonin from the intermediate. The shikonin produced is known to be secreted onto the cell wall (Shimomura *et al.*, 1991 ; Tsukada and Tabata, 1984). **Figure 4** shows that the amount of shikonin secreted was much larger in the

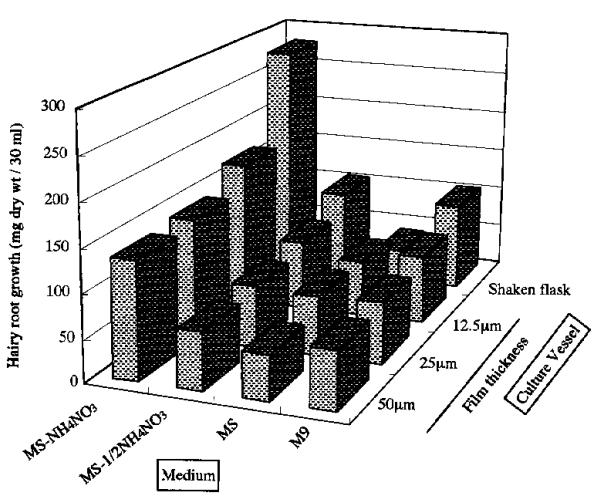

Fig. 2 The effect of the film thickness and type of medium on the growth of *Lithospermum erythrorhizon* hairy roots cultured in Culture Bags and 100 m*l* flasks containing various types of medium (30 m*l*). The former was cultured on a net without shaking and the latter was on a shaker at 70 rpm in the dark at 25°C for 3 weeks.

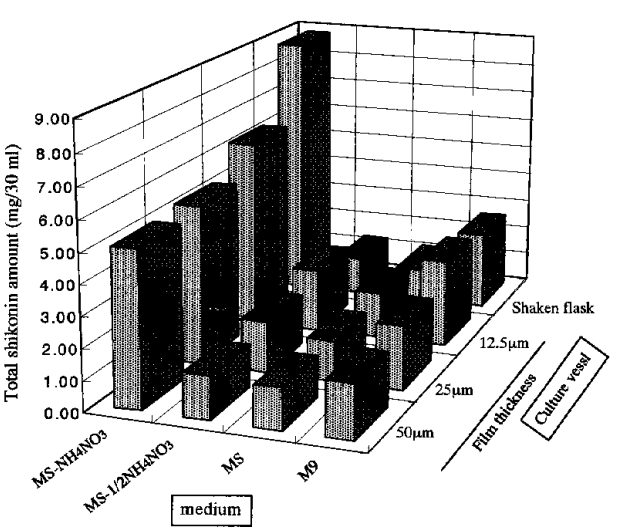

Fig. 3 The effect of the film thickness and type of medium on shikonin production in *Lithospermum erythrorhizon* hairy roots cultured in Culture Bags and 100 m*l* flasks containing various types of medium (30 m*l*). Culture conditions are the same with those of **Fig. 2**

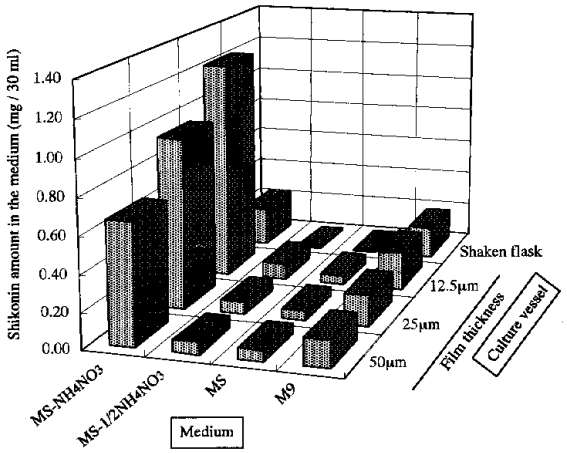

Fig. 4 The effect of the film thickness and type of medium on shikonin secretion in *Lithospermum erythrorhizon* hairy roots cultured in Culture Bags and 100 m*l* flasks containing various types of medium (30 m*l*).
Culture conditions are the same with those of **Fig. 2**

Culture Bag than in the shaken flasks containing MS $-NH_4NO_3$ medium; the ratio of shikonin secreted is 13~20% of the total shikonin in the Culture Bags and 2% in the shaken flasks. The larger secretion of the metabolites by the hairy roots in the Culture Bag than in the shaken flasks suggests that the hairy root cultures could be effectively used, as shown by Shimomura *et al.* (1991), for continuously metabolite-removed culture-vessels containing such a stationary liquid medium as the Culture Bag. It is not known, however, whether shaking stress inhibits the secretion or breaks the hairy roots to small pieces that catch and adsorb the hydrophobic shikonin secreted.

The present study together with our previous report (Fukui and Tanaka, 1995) indicates that the Culture Bag is useful for the production of secondary metabolites by plant cell and hairy root cultures without agitation as well as for studies of the physiological effect of shaking or agitation on plant tissue and cell cultures in a liquid medium.

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