Growth of Callus Tissue of *Catharanthus roseus* in Suspension Culture

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The successful suspension culture of *Catharanthus roseus* (L.) G. Don callus tissue for an indefinite period of time has been accomplished. The growth rates of the suspension cultures, which were grown in media with and without growth substances, are reported. Alkaloids were detected in both the tissue and the media.

THE IMPORTANCE of *Catharanthus roseus* (L.) G. Don (*Vinca rosea* L.) as a source of anticancer alkaloids is well established (1). The report of the 1964 Symposium on the Chemistry and Biological Activity of Catharanthus, Vinca, and Related Indole Alkaloids (2) provides considerable information on this subject. The literature also contains reports on the growth of *C. roseus* as a tissue culture (3–7) and of the presence of alkaloids in *Catharanthus* callus tissue. Previous tissue culture studies of *C. roseus* have been concerned with growth on solid media. This paper reports a study of *C. roseus* tissues grown in suspension culture over a period of several months.

EXPERIMENTAL

Plant Tissues and Media.—Callus cultures of *C. roseus* which had been growing on solid media for more than 3 years provided the source of tissue for initiation of suspension cultures. The solid nutrient medium was a modified White's formula containing 15% coconut water and 3 mg./L. of 2,4-dichlorophenoxyacetic acid (2,4-D) (3). A preliminary study revealed that while a modified White's medium provided excellent nutrition for the growth of *C. roseus* tissues cultured on solid media, a modified Wood and Braun medium (5) was preferable for suspension culture. The modified Wood and Braun medium as reported previously (5) was used as a basal medium throughout this study.

Inoculation, Growth, and Transfer Procedures.—Suspension cultures of *C. roseus* were initiated by aseptically inoculating 100 ml. of Wood and Braun's modified medium contained in 500-ml. conical flasks, with approximately 3 Gm. of callus tissue which had been growing on solid media. The inoculated flasks were placed on an Eberbach rotating shaker which operated at 160 r.p.m. on a 1.5 in. circle. The shaker was located in a dark room maintained at a temperature of 26°. Once suspension cultures were successfully established these tissues were used as inocula for initiating continuing suspension culture studies. A circular metal scoop with a perforated base, 1 cm. in diameter and 0.7 cm. in depth, was constructed and used to transfer suspension cells to fresh liquid media. The scoop was attached to the handle of a conventional inoculating needle and the unit was easily sterilized by flaming. Numerous transfers of tissue were made and their weights determined in order to find the approximate average weight of tissue contained in one scoop. The average figure was found to be 255 mg.

In an attempt to determine the effect of naphthaleneacetic acid (NAA) and kinetin on the growth of continuing suspension cultures, two media formulations were studied. One medium consisted of Wood and Braun's basal medium without the presence of either kinetin or NAA. A second formula contained the basal medium plus NAA, 1 mg./L., and kinetin, 0.5 mg./L.

The procedure for inoculating and subsequently continuing growth of the suspension cultures was the following. The appropriate medium was prepared and adjusted to pH 5.8 and placed in conical flasks (100-ml./500-ml. flask). The flasks were plugged with cotton and sterilized by autoclaving for 15 min. at 15 p.s.i. One hundred milliliters of media was inoculated by the aseptic addition of five scoops of suspended cells (approximately 1.275 Gm.). The inoculated flasks were placed on a rotating shaker. The suspension cultures were observed daily and at the end of the growth period the flasks were removed from the shaker. At that time some of the suspended cells were aseptically removed for the purpose of inoculating new media. The remainder of the suspended cells were collected on filter paper as the cell-media suspension was passed through a Büchner funnel. The fresh weight of the tissue from each flask was determined and recorded. In each case the tissue which had been removed for inoculation of new media was included in the total fresh weight figure. The tissue which was collected on the filter paper was wrapped in aluminum foil and frozen for later chemical investigation. The results of the growth rate study are recorded in Tables I and II. As noted in these tables, five series of suspension cultures, each series consisting of from 10 to 20 cultures, were grown on media with NAA and kinetin, while 10 series of cultures were grown on the basal medium without the addition of any growth substance.

In a separate experiment suspension cultures of *C. roseus* were grown in 250-ml. conical flasks, some of conventional design and others containing three baffles in the base of each flask. The baffled flasks are a product of Bellco Glass Co. The purpose of this experiment was to determine if increased agitation and aeration of cells would have any effect on the growth rate. The 250-ml. conical flasks each contained 50 ml. of sterile Wood and Braun medium with kinetin, 0.5 mg./L., and NAA, 1 mg./L. A suspension culture inoculum of approximately 765 mg. was aseptically placed in each flask of sterile medium. Both the conventional and the triple-baffled flasks were placed on an Eberbach rotating shaker which operated at 160 r.p.m. Two growth cycles were evaluated and the growth rates of tissues

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TABLE I.—MODIFIED WOOD AND BRAUN MEDIUM WITHOUT GROWTH SUBSTANCES

Series	Flasks Inoculated	Flasks Contaminated	Days Cultured, No.	Av. Fresh Wt./Flask, Gm.[a]
1	20	3	47	19.361
2	10	5	53	15.324
3	20	8	48	17.913
4	10	3	41	18.534
5	20	7	41	24.936
6	20	6	53	19.899
7	20	0	41	12.920
8	10	2	41	21.578
9	20	0	53	11.415
10	20	4	54	14.581

[a] Growth rates were not determined if flasks were contaminated.

TABLE II.—MODIFIED WOOD AND BRAUN MEDIUM CONTAINING KINETIN AND NAA[a]

Series	Flasks Inoculated	Flasks Contaminated	Days Cultured, No.	Av. Fresh Wt./Flask, Gm.[b]
1	10	5	53	19.834
2	10	5	47	24.249
3	20	7	47	15.935
4	10	1	53	15.631
5	20	8	53	10.044

[a] Kinetin in concentration of 0.5 mg./L. and NAA in concentration of 1 mg./L. [b] Growth rates were not determined if flasks were contaminated.

cultured in the two types of flasks were compared. In each case the tissue was harvested after 54 days.

RESULTS AND DISCUSSION

It has been demonstrated that *C. roseus* tissue cultures can be grown under submerged conditions and it appears, that providing proper subculturing procedures are performed, growth can be carried on indefinitely. In the experiment employing growth substances, the concentration used in liquid media was considerably less than that employed previously in solid media. In fact, it was found in one experiment with suspension cultures that the tissue required no added growth substance in the medium. The mean fresh weight, per flask, of tissue grown in a medium without NAA and kinetin was 17.646 Gm. while the average fresh weight of tissue grown on a medium with these growth factors was 17.938 Gm. In each instance the weight of inoculum was about 1.3 Gm. Several previous attempts to maintain *C. roseus* tissue cultures on solid media without growth substances met with only limited success. The tissue grew very slowly, especially in comparison to tissue grown on media with growth substances.

In the flasks of media without growth substances there was never evidence of tissue differentiation. Furthermore, on several occasions suspension cells were transferred to solid nutrient media without growth substances and in no instance was there evidence of differentiation which has been reported under similar circumstances with other tissues.

Despite the fact that the suspension cultures were conditioned to the media over a considerable period of time, very little growth appeared in the first 3 weeks after cells were transferred to new media. After this 3-week "lag" period growth occurred at a rapid rate for approximately 3 weeks. In flasks in which the cells remained as clumps or clusters, as opposed to those flasks in which the cells were finely dispersed, there was much slower growth. In a few flasks very little growth occurred and the clumps of cells at the end of the growth period appeared about the same as at the beginning. Problems of microorganism contamination were encountered as are indicated in Tables I and II. No growth rates were determined for flasks of tissue which became contaminated.

In the experiment concerned with cultures in conventional and baffled flasks it was found that suspension cultures in conventional conical flasks grew at a significantly faster rate than did cultures in the triple-baffled flasks. Apparently the increased agitation and aeration does not improve the growth rate but in fact may be detrimental to growth.

A qualitative examination of the tissues and the spent media from this study revealed the presence of several alkaloids in both the tissue and the media. The suspension culture of *C. roseus* is presently being scaled up from 500-ml. flasks to multiliter fermentors.

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