Short Communication Plant Tissue Culture Letters, **10** (2), 172-175 (1993)

Effects of the Addition with Various Adsorbents on the Protoplast Culture of Grape

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(Received January 5, 1993)
(Accepted February 16, 1993)

Previously, we succeeded in a gelling a culture of protoplasts derived form Kosyu strain of grape (*Vitis vinifera* L.) to form its callus[1]. In the culture, it was necessary to use Gellan gum with a large diffusion capacity as a gelling agent and to add an adsorbent, activated charcoal into the medium. The high division rate of the cell culture was considered to be due to the elimination of some inhibitors by activated charcoal. As part of the research into inhibitors of cell division, we made a screening of various adsorbents and found two effective substances, still not reported.

As the standard medium for the culture of protoplasts from callus tissues of Kosyu strain, MS[2] ($CaCl_2 \cdot 2H_2O$ at 1/5 concentration of the original) and 0.3% of Gellan gum as a gelling agent were used and each of the adsorbents described in **Table 1** was suspended in the medium for protoplast culture. The effect of the addition of each adsorbent on the development of protoplasts was examined. For isolation of protoplasts, about 2 g of fresh cell aggregates obtained by the suspension culture of callus tissues were transferred to a petri dish containing 10 m*l* of a solution containing 1.5% Cellulase Onozuka RS (Yakult), 0.1% Pectolyase Y-23 (Seishin Seiyaku), and 0.4 M mannitol in 0.1 M MES buffer (pH 5.5). The number of protoplasts in the medium was adjusted to an initial concentration of about 1×10^5 cells/m*l*. Other conditions for the preparation and culture of protoplasts are the same as mentioned previously[1].

The supernatant prepared by centrifugation at 370×g for 10 min. from the callus suspension after two weeks culture obtained as previously[1], was treated or not with an adsorbent and then the effect of the addition to the protoplasts culture (0.3% gellan gum added as a gelling agent, 4 m*l* in total per petri dish; 60×15 mm) on the frequency of cell division was investigated. The adsorbent-treated supernatant was prepared as follows. One percent (W/V) of an adsorbent was added into the supernatant and then it was allowed to stand for 90 min. with occasional stirring. Thereafter, the adsorbent was filtered off through a filter paper and the filtrate was further passed through a filter (0.2 μm, pore size) to eliminate bacteria and this filtrate was used as a sample.

The effects of the test adsorbents on the frequency of cell division were investigated. The results were shown in **Table 1**. It was indicated that Amberlite XAD-4 and bentonite have an effect similar to activated charcoal. Judging from the finding on the property of Amberlite XAD-4 and bentonite, application of these adsorbents to cultures of other plant species would be expected. The remaining adsorbents exhibited almost the same effect as the blank control, whereas activated alumina and

Table 1. Effects of some adsorbents on the development of protoplasts from grape cell culture.

Adsorbent*1	Concn. (%, W/V)	Frequency of cell division after 10 days (%)	
		Mean	Range
Activated charcoal	0.03	67	45-81
	0.05	83	68-94
	0.10	77	66-94
	0.20	68	41-88
	0.40	32	20-62
Bentonite	0.01	77	56-92
	0.04	39	22-72
	0.08	16	0-27
Amberlite	0.20	18	0-39
	0.50	46	34-52
	1.00	58	30-74
	1.20	55	41-80
Celite	0.05	11	7-17
	0.20	17	10-22
Alumina	0.05	1.9	0-12
Hydroxyapatite	0.05	3.2	0-16
Silica gel	0.05	21	4-26
	0.20	16	0-28
PVP*2	0.05	7.7	0-19
	0.20	4.7	0-17
Polyclar AT	0.05	6.3	0-19
	0.20	2.8	0-19
PVPP*3	0.05	8.4	0-24
	0.30	13	0-30
Blank		14	0-24

The frequency of cell division was counted by microscopic inspection at three arbitrarily chosen points of 0.5 cm^2 per dish for 4 replicates. The division rate was determined as follows, No. of cells that divided more then once/No. of cells inoculated initially $\times 100$ (%), according to the method in the previous paper[1].

*1 Activated charcoal (obtained from Okuno, Osaka), Amberlite XAD-4 (Organo), Bentonite (Nacalai tesque), Activated alumina 200 (Nacalai tesque), Celite (Standard supercell, Nacalai tesque), Hydroxyapatite (Seikagaku Kogyo), Silica gel 60 (Nacalai tesque), Polyclar AT (Wako).

*2 Polyvinylpyrrolidone (Nacalai tesque).

*3 Polyvinylpolypyrrolidone (Sigma).

hydroxyapatite showed inhibitory effects. The optimum concentrations of the three effective adsorbents, activated charcoal, Amberlite XAD-4 and bentonite were 0.05, 1.0 and 0.01%, respectively. However, addition of these adsorbents at higher concentrations seems to cause a reduction in the division rate owing to the adsorption of some essential medium constituent or a physical hindrance.

Previous reports[3-7] suggested that the division inhibitor to be adsorbed, are phenolics, impurities contained in agar, or plant hormones in the case of other plant species, however, the substance in the present study has not been identified. Such inhibitory substances are supposed to be produced

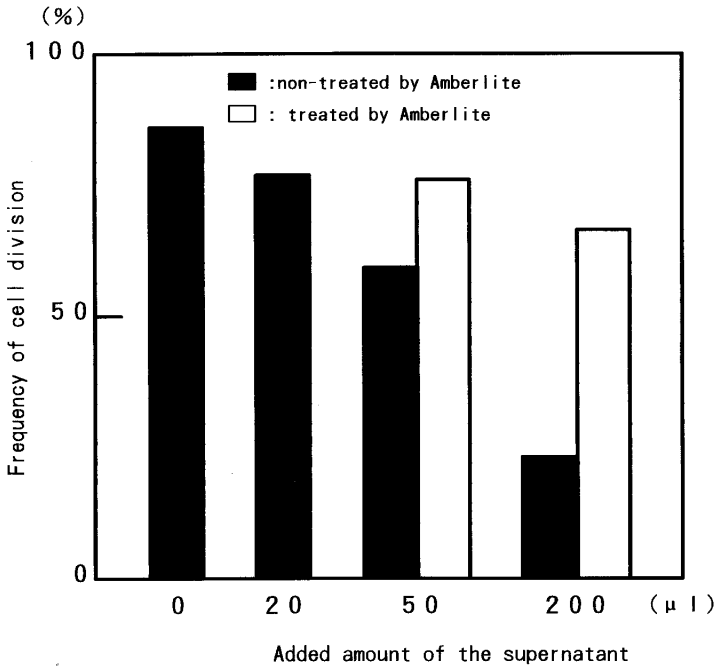

Fig. 1 Effect of addition of the supernatant treated or not treated by Amberlite on the frequency of cell division.
The figure was described on the basis of mean values of 4 replicates. Activated charcoal (0.05%) was added to each medium.

not only by the protoplast but also by the callus, although these should not necessaarily be looked on as identical. Various amounts of the adsorbent treated or not treated supernatant described above were added to the protoplast culture containing activated charcoal and the effects of the addition on the division rate were investigated. As shown in **Fig. 1**, the division rate was found to fall with an increase in the amount of the added supernatant. This result suggests that the callus culture would be so contaminated with inhibitory substances that the amount of activated charcoal added at the concentration of 0.05%, which is the usual one for the ordinary protoplast cultre, would be insufficient to eliminate them. When the supernatant to be added was treated in advance with an adsobent, the division rate was also recovered considerably. This was true even when Amberlite XAD-4, which is estimated to have the least adsorption capacity per weight from the results of **Table 1**, was used.

The presence of several effective adsorbents suggests that each adsorbent would have a characteristic intensity of absorption and conditions for its eluation. In that case, it seems that they would be convenient not only from a viewpoint of culture, but also for advancement in the identification of adsorbed inhibitors of cell division. Further, it was suggested that the culture medium of the callus cells is available for the investigation of sources of inhibitors of cell division. On the other hand, bentonite has a large base exchange capacity and a tendency to adsorb univalent cations[8]. These characters of bentonite are useful for the identification of such inhibitors.

Since phenolics produced in culture generally inhibit cell division in woody plants[9], it is said that the elimination of phenolics from a culture is necessary and that polyvinylpyrrolidone, polyvinylpolypyrrolidone and Polyclar AT are helpful for their elimination[3,9,10]. However, these three compounds manifested no effect in the present study and thus, the cause of the inhibition is thought to be some other substance (s).

Acknowledgements

In the beginning, we found that synthesized sand for disposition of cat's excreta (Hojyun-sand E) produced by Hojyun Co., Ltd (Osaka). has a marked stimulating effect on cell division of the protoplast. This finding gave us a chance to discover the above described effect of bentonite. So, we wish to express our thanks to Hojyun Co., Ltd. for kindly providing the information on the constituents of the sand. The authors also wish to thank Mr. Suzuki M. & Mr. Yazaki S. for their technical assistance in many of the analyses.

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《和文要約》

ぶどうプロトプラスト培養における
種々の添加吸着剤の影響

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甲州種ぶどう由来プロトプラストの培養には，活性炭以外にベントナイト及びアンバーライト XAD-4 についても同様の添加効果があることが新たに判明した．

一方，ポリビニルピロリドン類には全く効果が見られないことから，ぶどうプロトプラストの分裂阻害物質は，フェノール化合物とは異なる物質である可能性が高い．