

Tissue Culture in Mulberry Tree

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In recent years the method of *in vitro* cultivation of excised organs and tissues has been applied to the study of shoot and root growth of woody plants by several investigators. The applications of tissue culture are not only limited to solving the problems of plant physiology but also considered as an important means to develop the plant breeding method.

In Japan and India, the haploid plants have been obtained by the pollen and anther cultures, and these methods may provide a new approach for the promotion of plant breeding.⁵⁾

There are several investigations on the methods of culturing isolated tissues and organs of woody plants. In France Nitsch⁶⁾ succeeded in the culture of excised ovaries and tissues of fruit trees. This was the first attempt of the culture of fruit tissues *in vitro*.

In 1968 Winton⁸⁾ observed new buds and roots produced in the culture of callus tissues of aspen, obtaining the plantlets. It can be said that the way for producing genetically identical trees from single cells has been opened by this success.

In our laboratory the investigations were begun on the culture of excised tissues and organs of the mulberry tree for solving the problems of physiology, morphogenesis and breeding, and some results have been obtained. In this report the culture method of isolated roots, callus, and isolated buds is introduced.

Excised root culture in mulberry tree

Although isolated root tips of many plants

have been grown successfully (reviewed by Butcher and Street 1964)¹⁾, there are only a few examples of success in trees. Bonner was able to culture the root tips of acacia but failed to grow the roots of 12 other woody species. In Japan we have only one example of successful culture of isolated pinus densitron roots.³⁾

We investigated, as a part of the study of physiology of roots in woody plants,⁷⁾ the nutritious substances required for the growth of roots of the mulberry tree *in vitro* by the following method: At first, in order to obtain germ-free mulberry seeds, their surface was sterilized in a solution of sodium hypochlorite. After thoroughly rinsing and soaking in sterile water, the seeds were germinated on 0.6% agar in Petri dishes. Excised root tips (5 mm in length) were grown in 125 ml Erlenmeyer flasks each containing 30 ml of White's nutritious medium. The isolated roots were grown well at 28°C in darkness as shown in Fig. 1.

In the case of the mulberry tree it was found that the isolated roots can be grown in a relatively simple medium containing inorganic salts, carbohydrates, vitamins and hormones. In the early period of culture, thiamine and pyridoxine were not needed for satisfactory growth of root tips. However, when subculture of the main axis of root tips was attempted, it was observed that the meristematic activity in the individual root tips can be prolonged by adding thiamine and pyridoxine to the medium.

Moreover, the response of isolated mulberry roots to IAA was similar to that of pine roots. Namely, in case the concentration of IAA is above 10^{-5} mol, the elongation of roots and

initiation of lateral roots were inhibited and dichotomy was caused. On the other hand, application of IAA of low concentration (below 10^{-8} mol) stimulated the development of lateral roots.

By use of the culture method mentioned above, it may be possible to clarify what kinds of substances produced in the leaves would be most effective for the growth of roots in mulberry trees after harvesting.

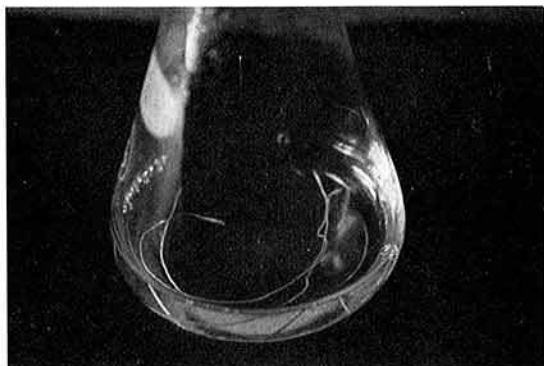


Fig. 1. Excised mulberry roots grown for 20 days in White's medium.

Callus culture in mulberry tree

The capacity of callus tissues to form organs was noted by Segretain who observed the formation of shoots in the culture of tobacco tissues, Skoog confirmed these results and reported that the callus tissues are capable of forming stem buds.²⁾ Recently Winton⁸⁾ succeeded in producing stem buds in aspen by the tissue culture. This is the first report on the isolation of plantlets in woody plants. In the case of aspen, leafy shoots were initiated on the subcultured callus tissues of triploid quaking aspen on the Wolter's medium with 6-benzylaminopyrine.

In our experiments with mulberry tree, root formation in callus obtained from tissues of root stocks and hypocotyls was observed, but formation of new shoots has not been recognized.

The callus tissues were obtained in our experiments as follows: The root stocks of 1-year old tree were cut into small sections

4-5 cm long and sterilized with 75% ethyl alcohol. Then the epidermis was carefully removed with a sterilized scalpel and the longitudinal slices taken from the cambial area or the segments 1 cm long were excised aseptically.

The segments were planted in Petri dishes containing the Murashige-Skoog's agar medium composed of 1 mg/l of 2,4-D. After 2 weeks the callus was isolated and subcultured on the same medium. In the early stage the isolated callus tissues grew without organ formation, but in the later stage the root initiation was observed. Fig. 2 shows the

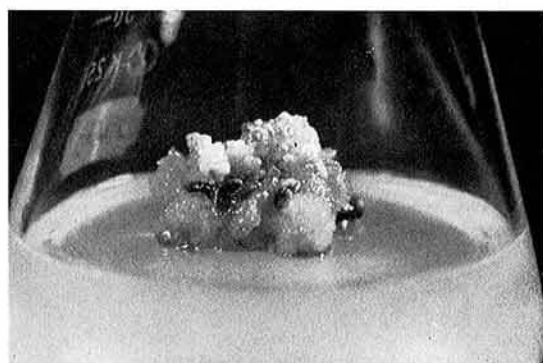


Fig. 2. Isolated mulberry callus tissues grown for 30 days in Murashige-Skoog's medium containing 2, 4-D 1 mg/l.

general appearance of non-differentiated callus tissues obtained from the mulberry root-stocks. The response of mulberry callus to auxin was similar to that of plant tissues in general.

The addition of 2,4-D to the basic culture medium accelerated the inducement and growing of callus, and the addition of NAA or IAA (1 mg/l) promoted the formation of roots. Fig. 3. These phenomena are very interesting for the study of propagation in mulberry tree, especially concerned with the problem of rooted cuttings.

Further, we have succeeded in culturing cell suspension on the callus tissues derived from the hypocotyl of mulberry seedlings in the following way: At first 100-200 mg of callus tissues were put into 100 ml flasks containing 25 ml of a liquid of Murashige-Skoog's



Fig. 3. Differentiation of roots from mulberry callus tissues.

medium, and then they were grown with shaking at 120 shakes per min. Fig. 4 shows the free cells of mulberry tissues thus obtained. These cells grew well in darkness in the synthetic media supplemented with 2,4-D (1 mg/l) and NAA (1 mg/l).

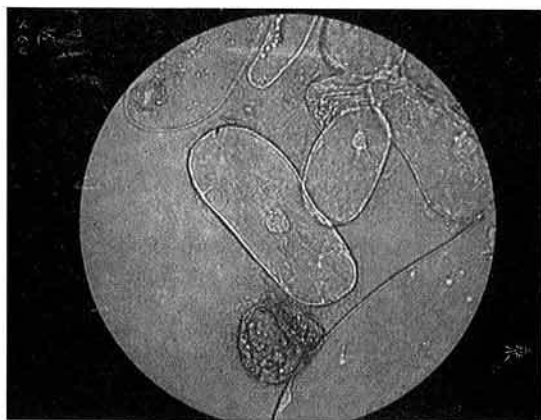


Fig. 4. Single cells of mulberry callus tissues as grown suspended in Murashige-Skoog's liquid medium containing 2, 4-4 1 mg/l.

In this case the total volume of cells was doubled in 7-8 days. A noticeable fact in the cell culture of mulberry tissues is that, as the cells are gradually deteriorated, yellow crystalline substances are formed, which is considered to be correlated with the phenomenon to become yellow in company with the growth of roots. Recently the cell culture has been applied for preparation of valuable biosynthetic products, and there is no doubt that the

study in this field must have a significance in its application.

Isolated bud culture in mulberry tree

Generally in plants, especially in woody plants, it is difficult to analyze the factors affecting the growth and dormancy of buds because of the existence of interrelations among the organs. Up to now several investigations have been made in this field on the culture methods by separating the buds from intact plants. For instance, Nekrasova¹¹ applied the method of culturing the isolated buds for studying the growth and development of fruit buds. In fact, such a method is very useful for analysis of the internal and external factors relating to the development of buds in woody plants.

We have also attempted to culture the isolated buds in the mulberry tree. The buds of a variety of mulberry trees including Kenmochi, Yôsô and Ichinose were used as materials. The buds to be tested were cut off from the shoots which had grown in the previous year.

The shoots were cut into small sections 4-5 cm long, and after carefully washing, these were sterilized by immersing in a solution of calcium hypochlorite for 30 minutes. Before planting in flasks the buds were put into sterilized cotton and the scales and bracts were carefully removed with a scalpel.

The planting was done in the 100 ml flasks each containing 20 ml of agar medium. Various nutritious media were used, but the Murashige-Skoog's medium was mostly used because it seemed to be most suitable for the culture of isolated mulberry buds. The medium was sterilized for 20 minutes in an autoclave at pressure 1 atm. All cultures were done at 28°C under artificial illumination.

Firstly, in order to examine the effects of some physiologically active substances on the opening and growth of isolated buds, four kinds of substances, i.e. 2,4-D, NAA, GA and Kn were added respectively to the medium at concentration of 0.1-1 mg/l. Of these, only

Kn stimulated the sprouting of buds and accelerated their growth, producing small stems with leaflets and 2,4-D enhanced the callus formation at the lower part of buds remarkably at all concentration, but the buds were scarcely opened. NAA and GA did not promote the sprouting of buds.

Secondly, the effects of the active substances in pairs were examined. The effects of 2,4-D and NAA used together were similar to those of GA alone. In the combination of Kn and NAA the growth of isolated buds began 7-8 days after planting and the growth of stem was observed, together with the unfolding of new leaves that had been present as leaf primordia. After 20-30 days new roots were formed at the lower end of stems. There was



Fig. 5. Opening and growth of isolated buds of mulberry on Murashige-Skoog's medium containing Kn 0.1 mg/l and NAA 1.0 mg/l.

a close relation existing between the formation of roots and the growth rate of stems. Rapid growth of stems and formation of new leaves were observed soon after the appearance of roots. This suggests that the activity of roots plays an important role for the growth of isolated buds Fig. 5, Fig. 6.

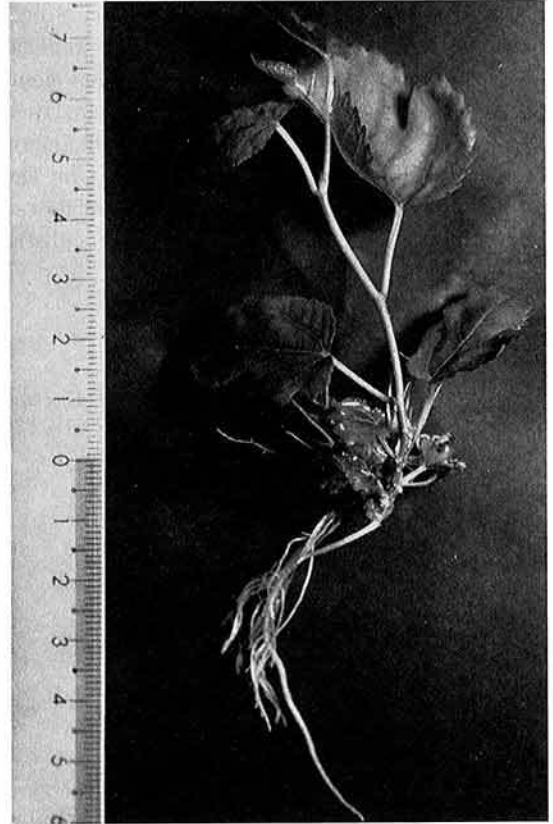


Fig. 6. Plantlets grown aseptically from isolated buds in mulberry tree.

Furthermore we observed that in a medium containing Kn adventitious buds were formed from epidermal cells of leaves. This fact is very noticeable because it was hitherto considered that the adventitious buds cannot be formed usually in the mulberry tree.

It was also observed that, on picking out flower clusters from the mulberry buds, the male and female flowers were normally bloomed. For the present, however, the pollination *in vitro* has not been successful yet.

Conclusion

The culture method of excised tissues, organs and cells has been described in the above. The tissue culture method will become an effective means in the future not only in the tree physiology but also in the practical problems of breeding, propagation, etc. Since, however, it requires a long time for general woody plants to attain to their efflorescence compared with herbaceous plants, a problematical point consists in the low breeding efficiency. It is, therefore, of great significance for the breeders, if it becomes possible to form flower buds in vitro by applying the tissue culture methods.

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