CULTURE OF GYMNOSPERM TISSUE IN VITRO

R. N. KONAR and CHITRITA GUHA

Department of Botany, University of Delhi, Delhi 7, India

SUMMARY

A review is given of recent advances in the culture of vegetative and repoductive tissues of Gymnosperms.

Gymnosperm tissue culture is still in its infancy as compared to its angiosperm counterpart. In spite of numerous efforts to raise cultures from time to time little success has so far been achieved.

1. CULTURE OF VEGETATIVE PARTS

Growth and development of callus

Success in maintaining a continuous culture of coniferous tissue in vitro was first reported by BALL (1950). He raised tissues from the young adventive shoots growing on the burls of *Sequoia sempervirens* on diluted Knop's solution with 3 per cent sucrose and 1 ppm IAA. Marginal meristems, cambium-like meristems around groups of tracheids and mature parenchymatous cells could be distinguished in the callus mass. The parenchymatous cells occasionally contained tannin. According to BALL (1950) anatomically the presence of tannin is not inconsistent with the normal function of the shoot apex. He considered that probably the tannin cells have less potentialities to develop.

REINERT & WHITE (1956) cultured the normal and tumorous tissues of *Picea glauca*. They excised the cambial region from the tumorous (characteristic of the species) and non-tumorous portions of tumor bearing trees and also cambium from normal trees. This work was carried out with a view to understand the degree of malignancy of the cells and the biochemical characteristics of the tumors. They developed a rather complex nutrient medium consisting of White's minerals, 16 amino acids and amides, 8 vitamins and auxin to raise the cultures. The tissues cultivated on filter paper moistened with this medium soon turned brown and eventually growth ceased. This was considered to be due to a copper containing enzyme – the phenoloxidase which appeared to be especially active in the traumatized tissue. When browning was prevented by the addition of tyrosine at a concentration of 40 mg/l or phenoloxidase inhibitors like diethyldithiocarbamate or sodium-ethylxanthate the tissues grew further for some time. However, only in tyrosine the growth was continuous. Substances like 2, 4–D, glutamine and IAA also enhanced growth.

They further noticed some differences in the behaviour of the normal and

tumorous tissues. The growth of the latter was faster but it was more difficult to establish them. The tumorous tissues could be grown much longer on a medium from which some amino acids or riboflavin and choline were omitted. Another striking characteristic noted was that the tumorous tissues appeared to require vitamin B_{12} which was not needed by the normal cells.

WHITE & RISSER (1964) have studied the conditions under which the best growth of the callus, derived from the tumor tissues of *Picea glauca* could be obtained. The maximal growth was achieved when 20 mg of the callus was inoculated on an agar substratum (0.5%) at pH of 5.5 in square bottles with screw caps. Cultures were maintained at 23°C either in darkness or in diffuse light and were transferred to fresh media every two weeks. Under these conditions the mean increment per passage was nine times.

In another publication (RISSER & WHITE 1964) their efforts were directed towards establishment of a medium less complicated than what had been suggested by Reinert & White in 1956. Sucrose at 5% concentration was found to be the most effective carbohydrate source which could be replaced satisfactorily only by dextrose. Fructose, mannose and raffinose were half as effective as sucrose. All the eighteen amino acids of the medium could be replaced by l-glutamine (250 mg or more/l). Only 4 vitamins could simulate the effect of 10 vitamins originally incorporated into the medium.

Amongst the auxins tried 10^{-7} M 2,4-D, 10^{-5}M IAA and 10^{-3}M β-naphthoxyacetic acid were effective growth promoters. However, 2,4–D was the best. Kinetin, adenine, β-alanine, gibberellin and folic acid in the presence of 2,4–D had no beneficial effect.

WHITE (1967) observed the cells of *Picea glauca* grown in vitro and reported certain processes which may contribute to the differentiation of distinct cell types recognizable in the plant. The cells exhibited certain patterned deposits in the cytoplasm which were in the form of strands, furrows or channels, that might act as templates for cellulose and lignin deposition. In some cells, the cytoplasm extruded into characteristic papillae and in others it was ejected slowly or with pressure. This condensed to form characteristic extracellular patterns.

To study the physiological differences in the tissues derived from plant parts or plants of different ages, BORCHERT (1967), as an initial step, determined the conditions necessary for in vitro culture of secondary phloem and cambium of *Cupressus lusitanica*. Explants proliferated well on a medium containing Heller's minerals and sucrose. When vitamins or growth substances were added to this medium, there was hardly any improvement in growth. However, the same substances, could considerably stimulate growth if added in Heller's medium containing higher concentrations of mineral salts.

HARVEY (1967) recently defined a medium for growing sucessfully the callus derived from the stem of *Pinus monticola*.

For approaching many problems in cellular biology, in recent years, suspension cultures have been found to be very useful. REINERT (1956) grew tumorous and non-tumorous tissues of *Picea glauca* and isolated a number of strains in which the normal tissue following particular change in the nutrient showed a

marked tendency to dissociate spontaneously and continued to grow with undiminished vigour. KONAR (1963a) studied the cell behaviour of the callus of *Pinus gerardiana* grown on Reinert's modified liquid medium. In suspension cultures, tissues dissociated into single cells which later divided to form cell aggregates.

2. BIOCHEMICAL STUDIES

2.1. Utilization of sugars

BALL (1955) reported the utilization of various sugars by the callus, obtained from the adventive shoots on the burls of *Sequoia sempervirens*. The normal chlorophyllous tissue contained glucose, fructose and sucrose. When the callus was grown on any of these sugars, the tissue could produce the other two. However, best growth could be achieved in a medium containing sucrose. When either raffinose or galactose was supplied as the source of carbohydrate, sucrose, fructose, glucose, galactose and raffinose were all detected in the callus. To explain this, Ball suggested that the enzyme that yields galactose from the hydrolysis of excess raffinose in the first instance forms raffinose in the second instance by condensation of excess galactose and sucrose.

2.2. Lignin formation

HASEGAWA *c.s.* (1960) demonstrated by means of radioactive compounds, the formation of lignin in the culture of cambial tissue of *Pinus strobus*.

They fed tissues with ^{14}C glucose, acetic acid and shikimic acid and analysed the four fractions (amino acid, acid, phenol and sugar) obtained, for radioactivity. They noted that when the tissues were fed with glucose-1-^{14}C maximum activity was in the acid fraction and this fraction contained shikimic acid. The extent of conversion of glucose to shikimic acid was found to be about 3–5 times more than that of acetic acid. The incorporation of acetic acid into shikimic acid was considerably low. From this, they concluded that shikimic acid and lignin are synthesized from glucose in the isolated cambium tissue. It was assumed that the aromatic nucleus of the lignin was formed from sugar via shikimic acid.

CONSTABEL (1965) showed the extent to which the tannin production in the cultures of *Juniperus communis*, depended on growth, on the presence of precursors and on light. He observed that with increase in growth rate there was a decrease in tannin content. Cinnamic, sinapic and ferulic acids were added to the medium to determine whether these compounds function as presursors of tannin or not. In their presence, a rise in the level of tannin was noted. These compounds stimulated growth at low concentrations but had an inhibitory effect at high concentration. Cinnamic acid was definitely thought to be a precursor of tannin whereas ferulic and sinapic acid could also function as precursors. There was a significant difference in tannin content of the tissues kept under 14 hrs light against those kept in dark. In dark, the tissues showed higher tannin production.

2.3. Growth factors

AL-TALIB & TORREY (1959) cultured the dormant terminal buds of *Pseudotsuga taxifolia*. It appeared that the isolated dormant terminal buds required oxygen, sucrose, glucose or fructose and light for development. They also studied effects of auxins, gibberellins, kinetin and adenine sulphate on the growth and development of the buds. Auxin at 10^{-6} M concentration had no stimulatory effect on bud development. At higher concentration (10^{-4}M), leaf development was generally retarded. The presence of gibberellic acid (0.1–1.0 ml/l) in the medium resulted in restricted leaf expansion and elongation of the main axis. Another effect was killing of the buds at later stages. Kinetin (0.001–1.0 mg/l) had a stimulatory effect on callus growth. Adenine sulphate reduced bud development and leaf expansion. Excellent bud development and leaf expansion were obtained when a seitz-filtered solution of urea was added to the medium at a concentration of 10^{-1}M. Supplements like coconut milk, yeast extract, casein hydrolysate and water melon juice were inhibitory to the development of the bud.

3. CULTURE OF REPRODUCTIVE TISSUE

Male cones of *Pinus roxburghii* at microspore mother cell stage, were cultured by KONAR (1963b) in White's medium supplemented with nucleic acids. The pollen mother cells underwent normal development giving rise to mature pollen grains. These were viable and germinated in vitro. Their anteridial cell divided to form the stalk and the body cell.

Pollen grains of *Ginkgo biloba* (TULECKE 1957), *Taxus* sp. (TULECKE & SEHGAL 1963) and *Ephedra foliata* (KONAR 1963) showed profuse proliferation in culture. This tissue is haploid and undifferentiated and could be compared to the male gametophyte of the lower plants. They are also of genetical interest being derived from microspores.

The pollen grains of *Ginkgo biloba* were cultured by TULECKE (1957) on modified White's basal medium with 0.25% yeast extract and 1 ppm IAA. The male gametophytes developed up to the immature sperm cell stage. Several abnormalities were, however, noted in the development of the male gametophyte. The most striking of these was the development of a basically haploid tissue. Biochemical and physiological studies were carried out to find the differences in the free and protein amino acids, sugars and non-volatile organic acids in the pollen and in the tissue derived from it. A strain of pollen tissue of *Ginkgo biloba* was isolated which required arginine (1000 ppm) for its growth (TULECKE 1960). Canavanine, an antimetabolite of arginine, inhibited the growth of the arginine requiring strain. Ammonia to a certain extent could replace arginine. TULECKE *c.s.* (1965) devised a set up termed "Phytostat" for obtaining continuous suspension cultures of *Ginkgo biloba* pollen tissue.

In 1963, KONAR reported the development of the pollen tissue of *Ephedra foliata*. Mature undehisced microsporangia were cultured in Reinert's modified medium supplemented with coconut milk and 2, 4–D. The pollen grains formed tiny masses of tissue. This tissue was haploid and showed various abnormalities.

TULECKE & SEHGAL (1963) apart from obtaining normal development of the male gametophyte in *Torreya nucifera*, also obtained a tissue from it. The majority of the pollen grains cultured showed abnormalities like division in the stalk and the sperm cell, extrusion of the generative cell etc. Often the tube cells divided by budding.

LARUE (1968) cultured the female gametophytes of *Zamia floridana* at various stages of growth on White's medium. Those cultured before fertilization showed little growth, whereas those at or after fertilization exhibited 5-fold increase in volume. On many gamethopytes suberized tissue was seen to develop and these eventually got covered by a loose layer of periderm. Only one percent of the cultures showed regeneration and produced either roots or shoots or both. The growth of the roots was restricted and they produced no secondary roots. Shoots developing from the primordia resembled the seedlings. These were believed to be the sporophytes but whether they were haploid or not could not be established. Development of a complete sporophyte, however, was not achieved. In a later paper LARUE (1954) reported that prolonged culturing produced spherical outgrowths which he called "pseudobulbils"; some of these grew out into plantlets.

NORSTOG (1965) working with *Zamia integrifolia* succeeded in inducing apogamy in the megagametophytes. He cultured them on White's basal medium with various growth promoting substances in 13 combinations. The highest percentage (59% after 5 months of culture) of regeneration of roots and leaves was observed on basal medium with kinetin 1 ppm, 2, 4–D 1 ppm, adenine 10 ppm, asparagine 100 ppm, glutamine 100 ppm and alanine 100 ppm. The regenerated organs were haploid and resembled those of the seedlings.

TULECKE (1964) reported the first success in obtaining a haploid tissue from the female gametophyte of *Ginkgo biloba*. The tissue formed was green and showed differentiation. He cultured the megagametophytes in modified Wood and Braun's medium. Microscopic examination showed the development of a number of tracheid-like cells in the tissue.

The proliferation in the female gametophytes of *Ephedra* in White's basal medium + 2, 4–D (5 ppm), kinetin (1 ppm), coconut milk (20%) and inositol (100 ppm) have been reported by SANKHLA *c.s.* (1967).

Studies on culture of embryos have been given much importance at the present times. This is useful for determining the factors influencing the development of embryos for overcoming dormancy and for rearing hybrid embryos. This technique has been applied to a few gymnosperms.

VASIL (1963) obtained callus from the embryos of *Gnetum* cultured at the "peculiar cell" stage on White's basal medium supplemented with casein hydrolysate or yeast extract (500 ppm). The callus comprised thin walled cells of various shapes and sizes. On subculture, after 4 weeks, it formed uniseriate filaments or small buds or proembryos. Attempts to induce differentiation of cotyledons in these proembryos were unsuccessful.

LOO & WANG (1943) grew one to several celled embryos of *Pinus yunnanensis* and *Keteleeria davidiana* on a basal medium (Pfeffer's solution + 2% sucrose +

0.6% agar) supplemented with heteroauxin (10 mg/l), nicotinic acid (1 mg/l) or vitamin B6 (0.1 mg/l). Addition of thiamin to the medium induced better growth of the embryos and they developed up to about 100 cells, while on a medium containing IAA the embryos could be reared to several hundred cells.

LI (1934) and BULARD (1952) cultured the excised embryos of *Ginkgo biloba*. LI reported that *Ginkgo* embryos could be grown in vitro with or without the cotyledons. However, growth of the cotyledons, hypocotyl and root ceased after about 15 days. BULARD, on the other hand reported that normal growth of embryos could only take place when the nutrient medium was in contact with the cotyledons, thereby serving a haustorial function.

SACHER (1956) grew excised embryos of *Pinus lambertiana* on White's basal medium with 2% sucrose on agar slants under continuous light. Embryos developed normally and the growth sequence paralleled those of in vivo. In contrast to the results of BULARD (1952), he found that normal development of the terminal bud took place even when the cotyledons were not in contact with the medium and therefore, he concluded that the haustorial function of the cotyledons could be bypassed in culture. He further observed, that when the excised embryos were planted with their radicle end submerged beneath the nutrient agar medium roots did not grow continuously.

The results of Sacher were confirmed to some extent by BROWN & GIFFORD (1958) and they also demonstrated the important role of cotyledons in affecting root growth of pine embryos grown *in vitro*. The excised embryos of *Pinus lambertiana* were reared in two ways. In one set of experiments, the radicle was introduced in the agar and in the other, the cotyledons were inserted. The tubes of the second set of experiments were kept inverted so that the roots were directed downwards. In the first experiments the initial root growth was rapid but after some time the growth was noticeably retarded. In the second instance, the growth of the root was more rapid and they reached a length of 5–8 cm. It was noted that when the plants were nourished through their cotyledons both rate and duration of root growth were better than when the nutrients were supplied via the root. In the next series of experiments the cotyledons were placed in a sucrose medium and the roots in a plain medium or *vice versa*. In both sets the roots were directed downwards. From these experiments they concluded that the manner in which sucrose was supplied to the excised embryo was important for the growth of the root.

GREENWOOD & BERLYN (1965), also working on *P. lambertiana*, established evidence of physiological polarity in sections of the embryos. The hypocotyls of the dormant embryos were cut into 2–3 mm segments and cultured in inverted tubes on Knop's medium containing 0.8% agar and 4% sucrose. When the segments were inoculated with their morphological basal end downward in air, roots emerged at this end. On reversing the orientation on root was formed.

BERLYN & MIKSCHE (1965) conducted several experiments with the embryos of *Pinus lambertiana* to study their growth behaviour in vitro, under various circumstances. They found that the cotyledons did not serve a haustorial function since even in their absence embryos could grow. In the next experiment, in

which the shoot meristems of the embryos were removed, there was an inhibition of the functional activity of the root meristem. They also carried out some inverted tube cultures to see the effect of position and light on growth. On placing such tubes in light in a vertical position better growth was observed than when they were placed horizontally. In dark the situation was reversed; cultures in a horizontal position showed an extremely high growth rate. Autoclaved coconut milk did not effect growth in any statistically significant manner.

In *Biota orientalis*, KONAR & OBEROI (1965) noticed the development of "embryoids" from the cotyledons of embryos grown on Butenko's medium. The embryoids developed into shoots when excised and transferred to fresh media. However, no root was formed.

Recently SANKHLA *c.s.* (1967) obtained callus from the root tips of excised embryos of *Ephedra foliata* in White's basal medium with 2, 4–D (1. 5 ppm) + CM 20%, when the callus was subcultured in White's medium + 2, 4–D (1 ppm) + CM 20% + kinetin 1 ppm embryoids developed spontaneously and differentiated into plantlets.

4. CONCLUDING REMARKS

A survey of the available literature on the culture of gymnosperm tissues in vitro indicates that despite the easy availability of material only a few plants have been utilized for such a study. Perhaps the only reason one can account for this is that gymnosperms are less amenable to cultural conditions and are extremely slow growing. In spite of considerable efforts only a few investigators have succeeded in maintaining a continuously growing callus. Differentiation of the callus has hardly been achieved.

An unsolved problem in biology is the phenomenon of differentiation. Nothing is yet known regarding the mechanism governing the process. As a preliminary step, BORCHERT (1967) has initiated studies on the physiological differences in the tissues derived from plant parts and plants of different ages.

Again, what governs the development of tumors, what is the biochemical nature of these tumerous cells and can they be reverted to normal tissues are some of the questions which have been pondered over at length. With a view to obtain answers to these questions REINERT & WHITE (1956) cultured the tumorous and non-tumorous tissues of *Picea glauca*. However, more work is needed to arrive at a conclusion.

The terminal buds undergoing a regular period of dormancy pose a difficult problem. AL-TALIB & TORREY (1959) attempted at solving it by culturing the terminal buds of *Pseudotsuga taxifolia*. They found that urea at a conc. of 10^{-1}M helps in breaking the dormancy to some extent. Tissue culture studies could thus be applied to determine the cause of this inactivity.

Haploid tissues have been raised from the pollen of *Gingko*, *Taxus* and *Ephedra* by TULECKE (1957), TULECKE & SEHGAL (1959) and KONAR (1963). Is it possible to differentiate these and what would be the nature of such plants? It remains to be seen.

The factors influencing the development of embryos in culture have been studied by a number of workers. This technique can be applied to successfully overcome seed dormancy and to rear hybrid embryos. Also, if the tissues of some economically important gymnosperms could be made to differentiate into plantlets some problems faced by the silviculturists could be solved.

Biosynthetic studies on tannin and lignin formation have been carried out by CONSTABEL (1965) and HASEGAWA *c.s.* (1960). Elucidation of pathways of lignin and tannin formation could help in determining the factors congenial to the continuous growth of the callus.

All the above-mentioned problems offer much scope for further work in this field.

REFERENCES

AL-TALIB, K. H. & J. G. TORRY (1959): The aseptic culture of isolated buds of Pseudotsuga taxifolia. *Pl. Physiol.* **34**: 630–637.

BALL, E. (1950): Differentiation in a callus culture of Sequoia sempervirens. *Growth* **14**: 295–325.

— (1955): Studies of the nutrition of the callus culture of Sequoia sempervirens. *Ann. Biol.* **31**: 281–305.

BERLYN, G. P. & J. P. MIKSCHE (1965): Growth of excised pine embryos and the role of cotyledons during germination in vitro. *Amer. J. Bot.* **52**: 730–736.

BORCHERT, R. (1967): The cultivation of tissues of Cupressus lusitanica in vitro. *Physiol. Plant.* **20**: 608–616.

BROWN, C. L. & E. M. GIFFORD, JR. (1958): The relation of the cotyledons to root development of pine embryos grown in vitro. *Plant Physiol.* **33**: 57–64.

BULARD, C. (1952): Culture aseptique d'embryons de Ginkgo biloba: role des cotyledons dans l'absorption du sucre et la croissance de la tige. *Compt. Rend. Acad. Sci. (Paris)* **235**: 739–741.

CONSTABEL, F. (1965): Phenolics in the tissue cultures derived from Juniperus communis L. Studies in tannin synthesis. In P. R. WHITE & A. R. GROVE (eds) *Internatl. Conf. Pl. Tissue Culture*. McCutchan Publishing Corporation, Calif. U.S.A., pp. 183–190.

GREENWOOD, M. S. & G. P. BERLYN (1965): The regeneration of active root meristems in vitro by hypocotyl sections from dormant Pinus lambertiana embryos. *Canad. J. Bot.* **43**: 173–175.

HARVEY, A. E. (1967): Tissue culture of Pinus monticola on a chemically defined medium. *Canad. J. Bot.* **45**: 1783–1787.

HASEGAWA, M., T. HIGUCHI & H. ISHIKAWA (1960): Formation of lignin in tissue culture of Pinus strobus. *Plant & Cell Physiol.* **1**: 173–182.

KONAR, R. N. (1963a): Studies on submerged callus culture of Pinus gerardiana Wall. *Phytomorphol.* **13**: 165–169.

— (1963b): A haploid tissue from the pollen of Ephedra foliata Boiss. *Phytomorphol.* **13**: 170–174.

— (1963c): In vitro studies on Pinus roxburghii Sar. In P. MAHESHWARI & N. S. RANGASWAMY (eds) *Plant Tissue & Organ Culture – A Symposium*. International Soc. Pl. Morphologists, Delhi pp. 224–229.

— & Y. P. OBEROI (1965): In vitro development of embryoids on the cotyledons of Biota orientalis. *Phytomorphol.* **15**: 137–140.

LARUE, C. D. (1948): Regeneration in the megagametophyte of Zamia floridana. *Bull. Torrey bot. Club* **75**: 597–603.

— (1954): Studies on growth and regeneration in gametophytes and sporophytes of gymnosperms. *Brookhaven Symp. Biol.* No. 6: 187–208.

LI, T. T. (1934): The development of Ginkgo embryo in vitro. *Science Rep. Nat. Tsing Hua Univ.* **2**(B): 41–52.

LOO, S. W. & F. H. WANG (1943): The culture of young conifer embryos in vitro. Science **98**: 544.

NORSTOG, K. (1965): Induction of apogamy in megagametophytes of Zamia integrifolia. *Amer. J. Bot.* **52**: 993–999.

REINERT, J. (1956): Dissociation of cultures from Picea glauca into small tissue fragments and single cells. *Science* **123**: 457–458.

— & P. R. WHITE (1956): The cultivation in vitro of tumor tissues and normal tissues of Picea glauca. *Physiol. Plant.* **9**: 177–189.

RISSER, P. G. & P. R. WHITE (1964): Nutritional requirements of spruce tumor cells in vitro. *Physiol. Plant.* **17**: 620–635.

SACHER, J. A. (1956): Observations on pine embryos grown in vitro. *Bot. Gaz.* **117**: 206–214.

SANKHLA, N., D. SANKHLA & U. N. CHATTERJI (1967a): In vitro induction of proliferation in female gametophyte tissue of Ephedra foliata Boiss. *Naturwiss.* **54**: 203.

—, — & — (1967b): Production of plantlets from callus derived from root tip of excised embryos of Ephedra foliata. *Naturwiss.* **54**: 349.

TULECKE, W. R. (1957): The pollen of Ginkgo biloba: In vitro culture and tissue formation. *Amer. J. Bot.* **44**: 602–608.

—, (1960): Arginine-requiring strains of tissue obtained from Ginkgo pollen. *Plant Physiol.* **35**: 19–24.

— & N. SEHGAL (1963): Cell proliferation from the pollen of Torreya nucifera. *Contrib. Boyce Thompson Inst.* **22**: 153–163.

— (1964): A haploid tissue culture from the female gametophyte of Ginkgo biloba L. *Nature* **203**: 94–95.

—, R. TAGGART & L. COLAVITO (1965): Continuous cultures of higher plant cells in liquid media. *Contrib. Boyce Thompson Inst.* **23**: 33–46.

VASIL, V. (1963): In vitro culture of embryos of Gnetum ula Brongn. In P. MAHESHWARI & N. S. RANGASWAMY (eds) *Plant Tissue and Organ Culture – A Symposium*, Internatl. Soc. Pl. Morphologists, Delhi. pp. 278–280.

WHITE, P. R. & RISSER, P. G. (1964): Some basic parameters in the cultivation of spruce tissues. *Physiol. Plant.* **17**: 600–619.

— (1967): Some aspects of differentiation in cells of Picea glauca cultivated in vitro. *Amer. J. Bot.* **54**: 334–353.