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TISSUE CULTURE OF ORCHIDS

I. METHODS FOR LEAF TIPS

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Summary

Leaf-tips of *Epidendrum* and *Laeliocattleya* (Orchidaceae) have been cultured *in vitro*. *Epidendrum* seedling leaf-tips form calli on Murashige–Skoog medium (MS) or after being transferred to Knudson C agar following prior growth on MS. Plantlets also develop from tips of young leaves of mature *Laeliocattleya* plants cultured in Heller's medium or in a solution specifically devised for orchid shoot-tip cultures. In Heller's medium plantlet development is preceded by callus formation whereas in the shoot-tip medium protocorm-like bodies are formed first. These tissues when subcultured from Heller's medium or the shoot-tip solution to Knudson C agar also produce plantlets which resemble orchid seedlings. The results are discussed in relation to the components of each medium, the requirements of germinating orchid seeds and protocorms, and the difficulties encountered in attempts to culture tissues of monocotyledonous plants.

Introduction

Clonal propagation of certain orchids, e.g. *Cattleya*, *Cymbidium* and *Dendrobium*, by shoot-tip culture is now a well-established technique (Morel, 1960; Kim, Kunisake and Sagawa, 1970). It is restrictive, however, in that the most important growing part of the plant has to be sacrificed. A method of propagation using other less valuable parts of the plant would clearly be advantageous. Also, it might extend the range of species that can at present be successfully propagated in culture. Successful culture of seedling leaf bases has been reported (Champagnat, Morel and Mounetou, 1970). Because of the considerable economic interest in clonal propagation of orchids, we have already described the purely technological and horticultural aspects of leaf-tip cultures (Churchill, Arditti and Ball, 1971; Churchill, Ball and Arditti, 1970; Arditti, Ball and Churchill, 1971; Ball, Arditti and Churchill, 1971; Churchill *et al.* 1971). In this paper, we describe the culture of excised leaf-tips of *Epidendrum* and *Laeliocattleya*. A paper dealing with root-tip cultures has been submitted for publication elsewhere.

Material and methods

Plant material

Leaf tips were removed either from aseptically grown 1-year-old *Epidendrum* cv. O'Brienianum seedlings or from young leaves on new growths of mature *Laeliocattleya* cv. Portia 'Mayflower' plants before development of the notch (Plate 1, Nos. 1–3). The latter were washed and surface-sterilized by immersion in aqueous saturated calcium

hypochlorite (Wilson, 1915) for 10 minutes, followed by washing in sterile distilled water.

Cultural procedures

Tips from young leaves of mature *Laeliocattleya* plants were cultured either in Heller's medium (H), modified by the use of 30 g/l kitchen-grade sucrose and supplemented with 1 mg/l 2,4-D and 0.5 mg/l 6-benzyl amino purine, or a medium (M) devised for culture of *Cymbidium* and *Cattleya* shoot tips (Ball *et al.*, 1971).

Epidendrum seedling leaf tips were cultured three to five per flask, in medium MS (Murashige and Skoog, 1962) modified by the deletion of adenine, niacin and pyridoxine and the substitution of kitchen-grade sucrose. The nutrients were sterilized through 0.22 μm or 0.45 μm pore diameter Millipore filters. Following sterilization, the nutrients were adjusted to 1 l with either sterile distilled water or autoclaved agar suspension. The pH was 5.5.

Calli or protocorm-like bodies were cultured on Knudson C medium (K) prepared as for seed germination (Knudson, 1946; Scott and Arditti, 1959).

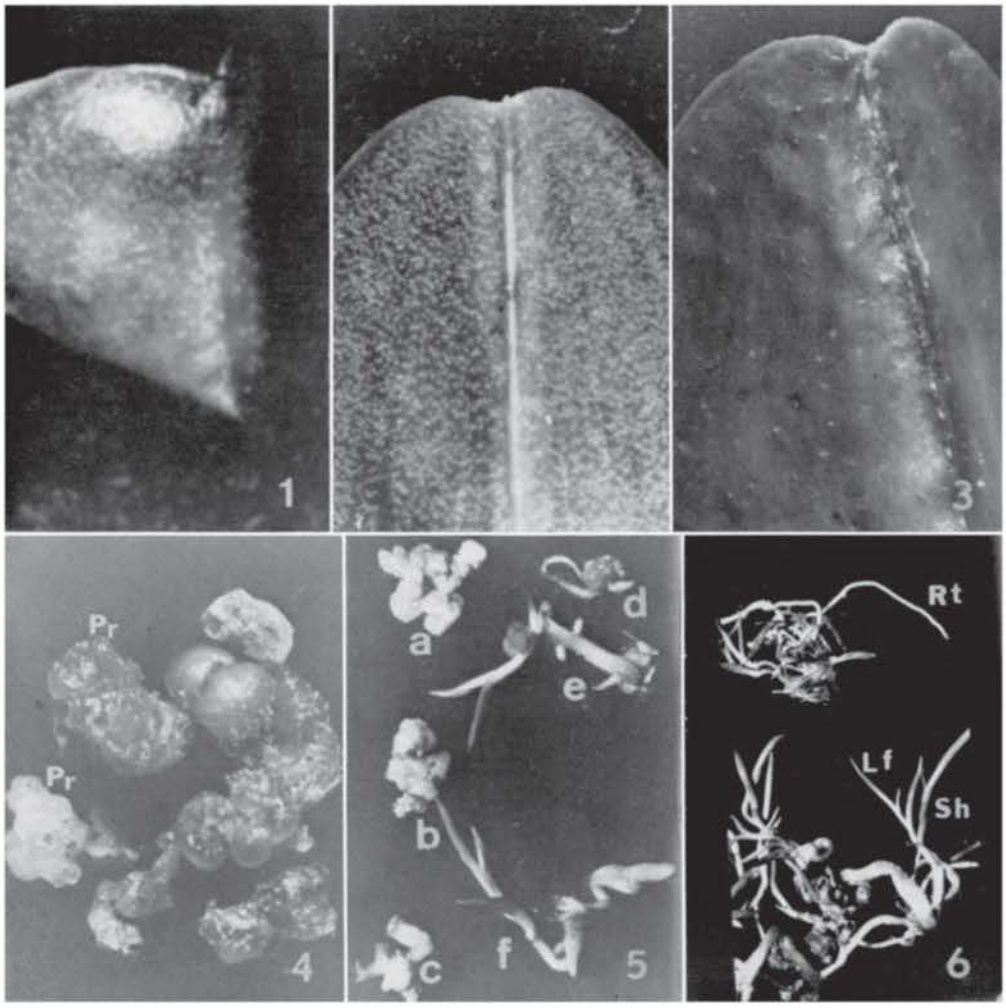
Liquid cultures were placed on a reciprocating shaker, adjusted to 60 oscillations/minute, 36 cm below a bank of Gro-Lux and incandescent bulbs giving an intensity of 150 ft-candles. Solid cultures were placed on a bench top under the same light-bank. Photoperiods were 18 hours. All cultures were maintained at laboratory temperature ($24+2^{\circ}$ C).

RESULTS

Although a large number of combinations of orchid species, organs and tissues with various culture media and additives was tested, success was limited. Callus cultures and, later, plantlets, were obtained only from leaf tips of *Epidendrum* seedlings and young leaves of mature *Laeliocattleya* plants. The latter developed protocorm-like bodies on liquid H and M media only (Table 1), whereas those from the former grew on MS agar only (Table 2).

In medium H, tips from young *Laeliocattleya* leaves remained green for up to 45 days without any apparent growth or external changes. Some died at the end of this period, but those remaining alive gave rise to small, green protocorm-like bodies or calli. The original explants died, but remained attached to the protocorm. After being transferred to a solid medium these grew very slowly for the first 10 weeks. Then, the growth-rate increased, several shoot apices were formed and, as each protocorm proliferated, a number of similar bodies were formed but remained attached to each other. Eventually, all protocorms formed leafy shoots and roots and developed into typical orchid plantlets which in many instances remained attached to each other. When separated, these grew into normal plantlets. In medium M, the formation of *Laeliocattleya* protocorm-like bodies followed the same sequence as outlined above. Once formed, these protocorms did not proliferate; some developed leafy shoots whereas others enlarged and their peripheries died. When transferred to medium K, the protocorms developed like normal orchid plantlets (Arditti, 1967).

Epidendrum seedling leaf tips, cultured in liquid medium MS, remained green without any apparent changes or growth for nearly 2 months. At that time, approximately 7% of the tips started to enlarge and, within 30 days, developed into calli (Plate 1, No. 4) which consisted of small green globules of tissue resembling a cluster of protocorms.



Key to lettering: Ca, callus; Lf, leaf; Pr, protocorm-like body; Rt, root; Sh, shoot.

No. 1. Young leaf from a mature. *Laeliocattleya* plant. Tip is still pointed. × 4. Excisions of the tip at this stage are capable of proliferation in culture.

No. 2. Young leaf from a mature *Laeliocattleya* plant. Notch is starting to form. × 4.

No. 3. Young leaf from a mature *Laeliocattleya* plant. Notch is well developed. × 4.3. At this stage the notch consists of mature parenchyma that is not dividing and as an excised portion it will not proliferate in culture.

No. 4. Callus formed from the leaf tips of an *Epidendrum* seedling. × 2.7.

No. 5. Development of a callus from the leaf tips of an *Epidendrum* seedling: (a) callus; (b) appearance of leaf tips on protocorm-like bodies of the callus; (c) formation of leaves; (d) flattened discoid callus on Murashige–Skoog medium; (e) and (f) plantlet formation on Knudson C medium. × 0.86.

No. 6. A mass of plantlets formed on a callus derived from an *Epidendrum* seedling leaf tip. × 1.75.

Each globule was 0.5–3 mm in diameter whereas the callus had a radius of approximately 1 cm. When shaken, the calli readily broke up into smaller clumps. These, after being subcultured on to solidified MS medium, began to proliferate again and 10–15 days later formed calli consisting of many globules (Plate 1, No. 4) which doubled in size within 3 weeks. Shaking broke up the clusters and each globule again formed a cluster-callus when placed on MS agar. This was repeated several times, always with the same results.

Table 1. *Macro-element and sugar content of media used for orchid-leaf-tip cultures (amounts expressed in μmoles)*

Nutrient	Knudson C (K)	Murashige–Skoog (MS)	Heller (H)	Medium (M)
Ammonium	8.40	20.62		
Nitrate	7.60	18.79	8.83	5.20
Phosphate	1.80	1.25	0.09	3.00
Sulphate	4.80	1.60	1.00	4.80
Potassium	1.80	20.00	10.00	7.00
Magnesium	1.00	1.50	1.00	1.00
Sodium		0.20	8.00	
Calcium	4.20	3.00	0.51	1.80
Chloride		6.00	11.00	
Iron	0.09	0.10	0.004	0.09
Manganese	0.034			0.034
Sucrose	58.43	87.64		29.21
Glucose			111.11	
Total nitrogen	16.00	39.41	8.83	15.20
Ammonium:nitrate ratio	1.11	1.097		
Total macro-elements	29.72	73.06	40.43	22.92
Total macro-elements plus sugar	88.15	160.70	151.54	52.23
Ranking, total concentration	3	1	2	4

Table 2. *Effects of culture media on orchid leaf-tips or callus cultures*

Orchid	Knudson C (K)	Murashige–Skoog (MS)	Heller (H)	Medium (M)
Epidendrum seedling leaf tips	Differentiation of callus into plantlets	Callus formation	No growth or callus formation	No growth or callus formation
Laeliocattleya, tips of young leaves of mature plants	Differentiation of callus into plantlets	No growth or callus formation	Callus or protocorm-like body formation followed by plantlet formation	Direct protocorm-like body formation followed by plantlet development

Two weeks following each subculture, some calli formed papillae-covered outgrowths which produced either new calli at their tips or flat and twisted leaves. Some green calli after being transferred again to MS agar developed into twisted, disoriented, rootless plantlets. These were transferred to medium K where, after 3 weeks of apparent inactivity, they developed into normal, rooted plantlets (Plate 1, Nos. 5 and 6). Roots were formed only on the new growth. If the multi-globular calli were transferred directly to K agar instead of to MS medium, they formed papillae-covered structures resembling large protocorms. Some calli or plantlets became red (probably owing to anthocyanins)

and others turned white. The white plantlets continued to develop normally, despite the lack of chlorophyll.

DISCUSSION

Although attempts were made to culture sections from most parts of the leaf (tips, petioles, various sections of the blade, base, parenchyma, etc.) on a great variety of culture media, only tips (Plate 1, Nos. 1–3) produced callus cultures, and only on the solutions listed. Since most angiospermous leaves show an early growth in length from what has been generally termed apical and subapical initial cells (Foster, 1936), it follows that there is probably such a meristem at the tip of the young orchid leaf that had proliferated in our cultures to produce a callus and later protocorms. Also, because leaf tips have been suggested as a source of mitotic figures for chromosome counts in orchids, it would seem that, as on other leaves, dividing and possibly meristematic tissue is present at that locale (histological studies presently in progress should help clarify this point). In our culture media, this tip-meristem tissue in the young leaf undergoes such numerous divisions that proliferations, comparable to protocorms, are formed.

In at least one orchid, *Malaxis paludosa* (L.) Sw. [*Hammarbya paludosa* (L.) O. Kuntze], buds naturally form on leaf tips and this character has been used in diagnoses and description of the species from Ray (1724) onwards. Therefore, the formation of calli and plants from leaf tips may merely reflect an inherent trait of the Orchidaceae which is brought out or 'turned on' by our culture media. *Malaxis paludosa* may represent an ultimate development in the Orchidaceae in which persistently meristematic tissue at the tips of leaves forms buds during development of the organ.

Media H, K and M were unsatisfactory for cultures of leaf tips from *Epidendrum* seedlings. Explants developed into calli only on MS. On the other hand, tips from young leaves of mature *Laeliocattleya* plants could be cultured only on media H and M. This may be due either to different requirements by leaf tips from seedlings or mature plants or to differences between genera (*Laeliocattleya* is a man-made intergeneric hydrid between *Cattleya* and *Laelia*).

MS and H are the most concentrated media used (Table 1) and the only ones to contain defined amounts of hormones and vitamins, although, as used by us, MS does not contain adenine, niacin and pyridoxine. M is more dilute but includes coconut milk which contains some vitamins and hormones. K, as normally used, is completely devoid or organic supplements, but is not as dilute as M (Table 1). Thus, either the known organic supplements in either H or MS or the high total concentrations (ionic plus sugar) of these solutions, or both may be responsible for callus formation. The major effect is probably that of total concentration. *Epidendrum* callus cultures when transferred to K, enriched with the micro-elements and organic supplements of MS, do differentiate (Hogan, Ball and Arditti, unpublished) but fail when subcultured on Linsmaier–Skoog medium (McIntosh, Ball and Arditti, unpublished), which differs from MS mainly in the concentrations of hormones, vitamins and amino acids (Linsmaier and Skoog, 1965). Furthermore K and M are similar in both inorganic salt content and total concentration (Table 1). M does contain coconut milk, a source of growth factors, yet differentiation occurs on both. All this suggests that variations in total concentration seem to be a major factor influencing differentiation.

Potassium concentrations do not have uniform effects on orchid seedlings (Arditti, 1967). Therefore, comparisons between the requirements of leaf-tip calli and protocorms are difficult if not impossible. However, it should be noted that MS contains

twice as much potassium as H and three times the amount in M. Possibly, therefore, high potassium concentrations may be a specific requirement of *Epidendrum* seedling leaf-tip explants.

MS and K contain ammonium ion, but H and M do not. Hence it would seem that it alone does not influence differentiation. Nitrate concentrations in K, H and M are lower than in MS which suggests that differentiation is not affected by this ion. Variations in concentrations of sulphate, phosphate, magnesium, calcium and iron also seem to be without effect.

Inasmuch as young *Laeliocattleya* leaf-tips developed calli on both H and M media it appears that the kind of sugar (sucrose or glucose) and its concentration do not play an important role in determining success or failure in their culture although optima, no doubt, exist. In this respect the requirements of young leaf tips seem to resemble those of seedlings (Arditti, 1967; Ernst, 1967; Ernst, Arditti and Healey, 1970, 1971).

It is noteworthy that successful tissue cultures of other monocots (rice and oats) were also accomplished on MS, whereas lily calli (Sheridan, 1968) were grown on the very similar Linsmaier and Skoog medium. That these relatively simple media can sustain monocot tissue cultures is considered somewhat surprising by some (Sheridan, 1968) in view of the difficulties and failures which are so common with this class. What we find surprising regarding orchid tissue cultures is not that successful cultures could be obtained on simple media since shoot tips are generally cultured on the K medium, which contain only mineral salts and sugar, but that, firstly, the percentage of leaf tips which do develop calli is low and, secondly, that other tissues have failed to respond to the addition of yeast extract, collagen hydrolysate, N-Z amine, enzymatic digest of casein or lact-albumin, acid casein hydrolysate (salt and vitamin free), banana homogenates or extracts (which greatly enhance seed germination and seedling growth) and coconut milk.

The production of plantlets from leaf tips is interesting because whole plants originate from a region which is destined to mature into foliar mesophyll and epidermis on the plant, thereby losing all meristematic capacity. In contrast, plants produced from shoot tips come from what is an essentially primordial stem tissue. The development of plants from shoot tips has been investigated (Morel, 1960, 1963, 1964, 1966; Champagnat, 1965; Champagnat *et al.*, 1966; Champagnat and Morel, 1969), but the process in the leaf tip is unknown. It is now being investigated in this laboratory. Comparisons between these two experimental systems are presently not possible, but the culture of young leaf tips is significant theoretically and practically in that it provides a new experimental system and a method of clonal propagation which is simpler than the use of shoot meristems and does not require the sacrifice of a plant, a shoot, a bud or even a whole leaf.

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REFERENCES

ARDITTI, J. (1967). Factors affecting the germination of orchid seeds. *Bot. Rev.*, **33**, 1.
ARDITTI, J., BALL, E. A. & CHURCHILL, M.-E. (1971). Propagacion clonal de orquideas utilizando apices de hojas. *Orquideologia*, **6**, 113.
BALL, E. A., ARDITTI, J. & CHURCHILL, M.-E. (1971). Clonal propagation of orchids from leaf tips. *Orchid Rev.*, **79**, 281.
CHAMPAGNAT, M. (1965). Particularités du dévelopement des apex *Cymbidum* cultivés *in-vitro*. *Bull. Soc. Franc. Physiol. Veg.*, **11**, 225.
CHAMPAGNAT, M. & MOREL, G. (1969). Multiplication végétative des *Cattleya* à partir de bourgeons cultivés *in vitro*. *Soc. Bot. Fr.*, Memoires, 111.
CHAMPAGNAT, M., MOREL, G., CHABUT, P. & COGNET, A.-M. (1966). Recherches morphologiques et histologiques sur la multiplication végétative de quelques orchidées du genre *Cymbidium*. *Revue gén. Bot.*, **73**, 706.
CHAMPAGNAT, M., MOREL, G., & MOUNETOU, B. (1970). La multiplication végétative des Cattleya à partir de jeunes feuilles cultivées aseptiquement *in vitro*. *Annals Sci. nat.* (*Bot.*), Sér. 12, **11**, 97.
CHURCHILL, M.-E., ARDITTI, J. & BALL, E. A. (1970). Clonal propagation of orchids from leaf tips. *Bull. Am. Orchid Soc.*, **40**, 109.
CHURCHILL, M.-E., BALL, E. A. & ARDITTI, J. (1970). Production of orchid plants from seedling leaf tips. *Orchid Dig.*, **34**, 271.
CHURCHILL, M.-E., FLICK, B., BALL, E. & ARDITTI, J. (1971). Vermehrung von Orchideen durch Blattspitzen. *Die Orchidee*, **22**, 147.
ERNST, R. (1967). Effects of carbohydrate selection on the growth rate of freshly germinated *Phalaenopsis* and *Dendrobium* seed. *Bull. Am. Orchid Soc.*, **36**, 1068.
ERNST, R., ARDITTI, J. & HEALEY, P. L. (1970). The nutrition of orchid seedlings. *Bull. Am. Orchid Soc.*, **39**, 599; **39**, 691.
ERNST, R., ARDITTI, J. & HEALEY, P. L. (1971). Carbohydrate physiology of orchid seedlings. II. Hydrolysis and effects of oligosaccharides. *Am. J. Bot.*, **58**, 827.
FOSTER, A. S. (1936). Leaf differentiation in Angiosperms. *Bot. Rev.*, **2**, 349.
KIM, K.-K., KUNISAKI, J. T. & SAGAWA, Y. (1970). Shoot-tip culture of dendrobiums. *Bull. Am. Orchid Soc.*, **39**, 1077.
KNUDSON, L. (1946). A new nutrient solution for germination of orchid seed. *Bull. Am. Orchid Soc.*, **15**, 214.
LINSMAIER, E. M. & SKOOG, F. (1965). Organic growth factor requirements of tobacco tissue culture. *Physiologia Pl.*, **18**, 100.
MOREL, G. M. (1960). Producing virus free cymbidiums. *Bull. Am. Orchid Soc.*, **29**, 495.
MOREL, G. M. (1963). La culture in vitro du méristème apical de certaines orchidées. *C. r. hebd. Séanc. Acad. Sci., Paris*, **256**, 4955.
MOREL, G. M. (1964). Tissue culture—a new means of clonal propagation of orchids. *Bull. Am. Orchid Soc.*, **33**.
MOREL, G. M. (1966). Clonal propagation of orchids by meristem culture. *Cymbidium Soc. News*, **20**, 3.
MURASHIGE, T. & SKOOG, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Pl.*, **15**, 473.
RAY, J. (1724). *Synopsis Methodica Stirpium Britannicarum*. Gulielmi and Joannis Innys, London.
SCOTT, R. J. & ARDITTI, J. (1959). Cymbidiums from pod to pot. *Bull. Am. Orchid Soc.*, **28**, 823.
SHERIDAN, W. F. (1968). Tissue culture of the monocot *Lilium*. *Planta*, **82**, 189.
WILSON, J. K. (1915). Calcium hypochlorite as a seed sterilizer. *Am. J. Bot.*, **2**, 420.