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Hormonal requirements for proliferation of pseudobulbils in citrus nucellus cultures

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Abstract. Continuous proliferation of pseudobulbils, presumed intermediary structures in citrus nucellar embryogenesis in vitro, was achieved by supplementing nutrient media with cytokinins, the kind and concentration varying according to species and cultivar. Those responding more favorably to BA, and the optima in M, were *Citrus aurantium*, 1.5 x 10^{-6}; *C. jambhiri*, 1.5 x 10^{-6} ; *C. reticulata* 'Cleopatra', 5 x 10^{-6}; *C. reticulata* 'Ponkan', 1.5 x 10^{-6}; and *C. sinensis*, 1.5 x 10^{-6}. Those for which 2iP was superior and the optimum M concentrations, were *C. sunki*, 5 x 10^{-5}; and the two monoembryonics, *C. grandis* 'Ma Dou Bai You', 1.5 x 10^{-5}; and *C. grandis* 'Ma Dou Wen Dan', 1.5 x 10^{-6} M. *C. limonia* and *C. macrophylla* required no hormonal supplements. None of the species or cultivars required auxin addendum, although *C. grandis* 'Ma Dou Bai You' showed significant enhancement of the 2iP effect by 5 x 10^{-7} M NAA.

Key words: *Citrus aurantium*; *C. grandis*; *C. jambhiri*; *C. limonia*; *C. macrophylla*; *C. reticulata*; *C. sinensis*; *C. sunki*; Nucellar embryo; Pseudobulbil; Tissue culture.

Introduction

Adventive embryo development from nucellus is a trait of many of the Rutaceae. The polyembryonic members produce seeds with few to several embryos each. In horticultural practice, citrus trees from nucellar embryos are sometimes desired for their renewed vigor and absence of most citrus viruses and virus-like pathogens. Seedlings of nucellar origin are also advantageous as clonal rootstocks for propagation by graftage. These advantages are not inherent in the normally monoembryonic species and cultivars, but can be duplicated through asexual embryogeny in excised nucellus cultured in vitro.

Rangaswamy (1961), in his pioneering studies of citrus nucellar embryony in vitro, observed that initial development from nucellus resulted in development of "pearly white, sometimes green, tumoroid outgrowths" that he identified as "pseudobulbils". The pseudobulbils were intermediary structures which, by an as yet undefined process, eventually gave rise to embryos. Embryo development from excised citrus nucellus through a pseudobulbil step has been confirmed by others, e.g., Sabharwal (1963) and Button and Bornman (1971) . The occurrence of citrus pseudobulbils in vivo remains unreported.

The pseudobulbils are easily distinguishable from callus. Their proliferation in vitro begins as small, globular outgrowths from surfaces of existing pseudobulbils (Fig. 1A); the outgrowths elongate (Fig. 1B) and eventually flatten out into clusters of green, cotyledon- and leaf-like structures (Fig. 1C). Plants arise after a period of culture or if left unsubcultured (Fig. 1D).

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[2] **Abbreviations:** AdS, adenine sulfate; BA, N^6-benzyladenine; ME, Difco Bacto malt extract; GA_3, gibberellic acid; 2iP, N^6-isopentenyladenine; NAA, 1-naphthaleneacetic acid.

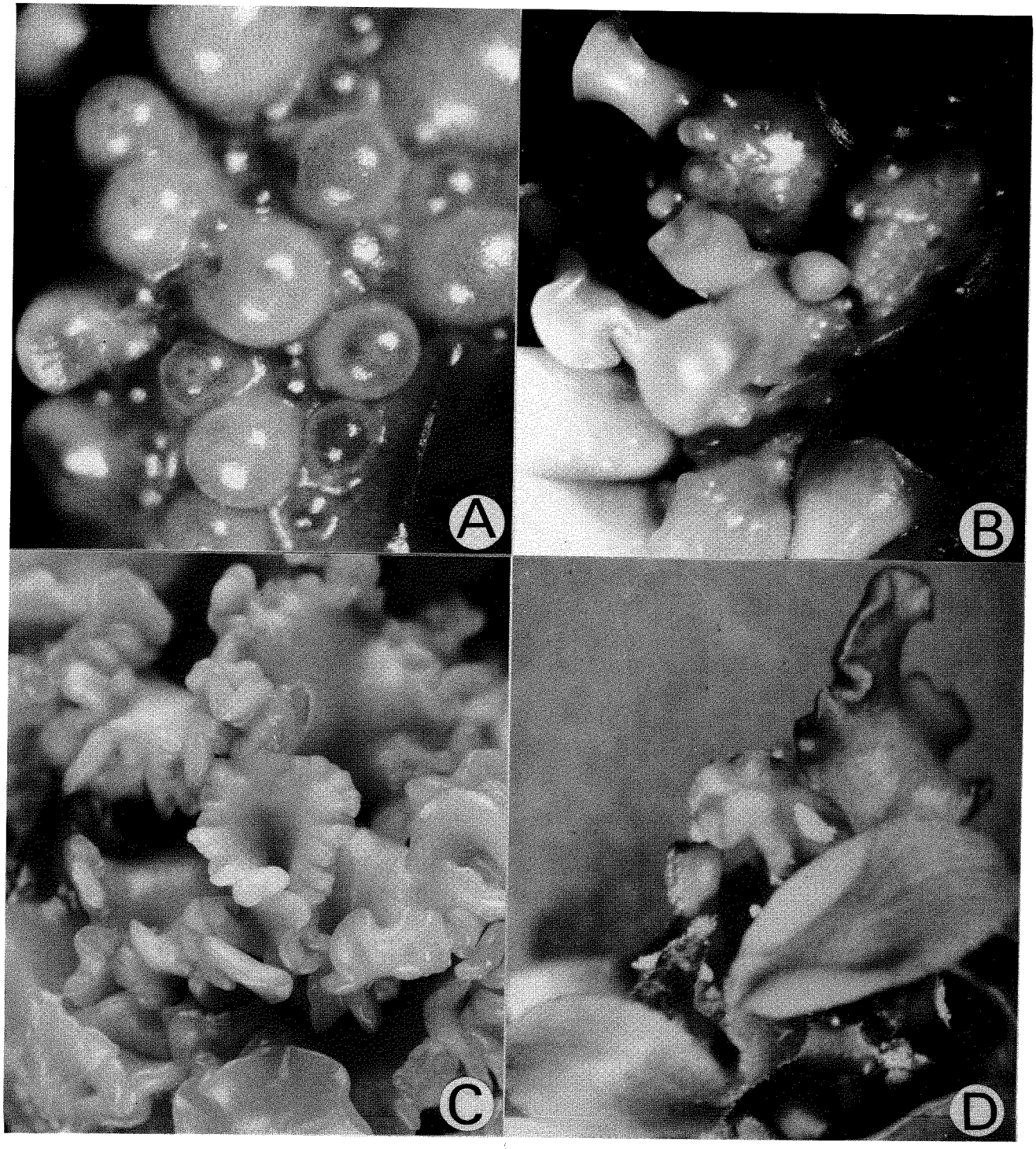

Fig. 1. Four stages of development of pseudobulbils of *C. jambhiri* nucellus culture. A. Initial stage of formation of pseudobulbils from surface of existing pseudobulbil (150X). B. Growth and early differentiation of pseudobulbils (75X). C. Fully differentiated pseudobulbils (30X). D. Plants emerged from pseudobulbils (12X).

This investigation began with the intent of utilizing pseudobulbils and nucellar embryos as sources of protoplasts for parasexual hybridization of citrus and somaclonal variants, as well as for virus elimination. The pseudobulbils were easily obtained by culturing nucellus explants in a medium supplemented with ME and AdS. However their proliferation and vigor could not be sustained without additions of cytokinin. Thus, the focus was on optimizing cytokinin supplements of selected species and cultivars, many of which are commercially important as rootstocks.

Materials and Methods

Species and Cultivars

The investigation examined these *Citrus* species and cultivars: *C. aurantium* L. (sour orange), *C. grandis* Osbeck cvs. 'Ma Dou Bai You' and 'Ma Dou Wen Dan' (shaddocks), *C. jambhiri* Lush (rough lemon), *C. limonia* Osbeck (Rangpur lime), *C. macrophylla* Wester, *C. reticulata* Blanco cvs. 'Cleopatra' and 'Ponkan' mandarins, *C. sinensis* Osbeck (sweet orange), and *C. sunki* Hort. ex Tan. The two shaddocks are monoembryonic, i. e., nucellar embryogenesis is not observed naturally. All others are polyembryonic and normally display varying degrees of adventive embryogenesis. Trees of the above were available in the germplasm collection of the Chiayi Agricultural Experiment Station, Taiwan Agricultural Research Institute.

Explant and Preparation

Young fruits with ovules containing zygotic embryos in the globular to heart-shaped stages were washed with detergent and as much of their flavedo and albedo removed to obtain small cubes with the ovules enclosed. The cubes were disinfested by immersing in a solution of 0.5% NaOCl and 0.1% Tween 20 emulsifier for 20 min, in vacuuo, and rinsing once with autoclaved water. Ovules were removed to sterile Petri dishes and, using the dissecting microscope, their nucelli excised. To excise the nucellus, the chalazal of the ovule was grasped with a small, fine-tipped forceps and a shallow incision was made through the integuments, longitudinally from chalazal to micropylar ends, care being taken not to injure the nucellus. The integuments were pried apart to expose the nucellus. The zygotic embryo was apparent as a glistening, yellowish-green hard structure, embedded in the micropylar region of the greyish, translucent and soft nucellus. The embryo was lifted out with the scalpel tip and discarded. The nucellus was then separated from the integuments and its chalazal half severed and also discarded. One micropylar half of nucellus was explanted per culture.

For the highly polyembryonic species and cultivars, 40 nucellar explants were employed to establish initial stocks of pseudobulbil cultures. The more moderately polyembryonic needed 60 explants and the monoembryonic cultivars, 80.

Subculturing and Experimenting with Pseudobulbils

In establishing stocks, only the green pseudobulbils and embryos that emerged from explants were recultured in fresh media. Any amorphous callus was discarded. For subculturing of stocks and experimenting, 1/2 g quantities of pseudobulbils were employed per culture. Stocks of polyembryonic *Citrus* were subcultured and experiments terminated after 4 wk, whereas those of the monoembryonic, because of slower growth, were subcultured or concluded after 8 wk.

Basal Composition and Media Preparation

The nutrient media contained these common ingredients: Murashige and Skoog (1962) salts, and in mg/L, sucrose, 30,000; *i*-inositol, 100; thiamine HCl, 1; pyridoxine HCl, 0.5; nicotinic acid, 0.5; glycine, 2; AdS $2H_2O$, 10; ME, 500; and Gelrite, 2000. The pH of all media was set at 5.7 prior to addition of Gelrite, using 1 N HCl or KOH. The media were dispensed in 25-ml quantities in 25- x 150-mm glass tubes, the tubes capped with Bellco Kaputs, and the media sterilized by autoclaving at 1.05 $kgcm^{-2}$ for 8 min and cooled as 30°C slants. Two cytokinins, BA and 2iP, were compared and the optimum cytokinin provision determined for each species and cultivar. One of the shaddocks appeared to also require auxin; hence, the cytokinin assays of the monoembryonic cultivars considered an additional supplement of 5×10^{-7} M NAA. Ten tubes of medium were employed per treatment.

Incubation Parameters

The cultures were incubated at 27°C constantly and under 16-hr daily exposure to 4.5 $nEcm^{-2}sec^{-1}$ illumination from Toshiba FL-40S-BR/38 fluorescent lamps.

Data and their Evaluation

The pseudobulbils could not be separated easily and counted accurately. Thus, the treatment effects were based on total fresh weight per culture. The data were evaluated by computing the standard errors of means according to Snedecor (1946). The large standard errors made interpreting figures difficult. Thus, data were plotted without the errors and only the statistically significant differences are cited.

Results

The cytokinin supplements increased and sustained proliferation of pseudobulbils of most species and cultivars through repeated subcultures. None of the media modifications affected embryogenesis or organogenesis, however, except for promotion of roots by NAA. The pseudobulbil yields are shown in Figs. 2 and 3.

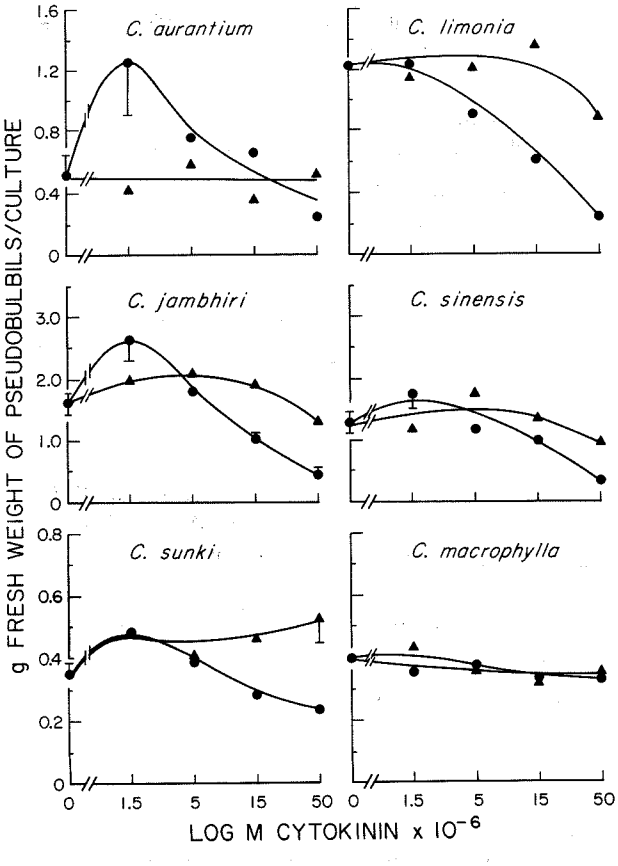

Fig. 2. Response of *Citrus* pseudobulbil cultures to supplements of BA (●), and 2iP (▲).

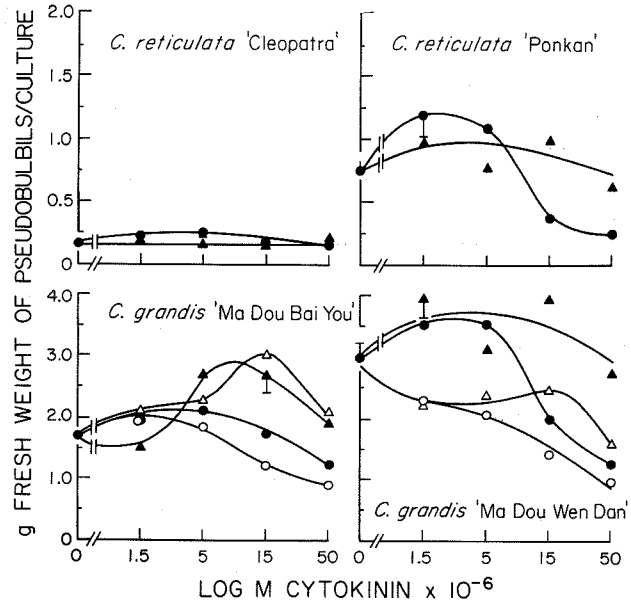

Fig. 3. Response of *Citrus* pseudobulbil cultures to supplements of BA (●), 2iP (▲), BA+NAA (○), and 2iP+NAA (△).

C. aurantium (polyembryonic). Statistically significant increase in pseudobulbils was obtained only with the 1.5 x 10^{-6} M supplement of BA. All levels of 2iP and other levels of BA were ineffective.

C. limonia (polyembryonic). Sustained pseudobulbil proliferation of this species did not require hormonal supplements. No significant increase in yield was obtained with either cytokinin. Pseudobulbils decreased with BA concentration. 2iP was less inhibitory, the pseudobulbil yield being suppressed only by its highest level, 5 x 10^{-5} M.

C. jambhiri (polyembryonic). Significant enhancement was obtained with a 1.5 x 10^{-6} M BA addendum. Higher concentrations were inhibitory; tissues became necrotic in the 5 x 10^{-5} M treatment. 2iP had no effect, except for a slight suppression at 5 x 10^{-5} M.

C. sinensis (polyembryonic). No statistically significant increase of pseudobulbil yield was recorded with either BA or 2iP. However, sustained vigor of subcultures required a supplement of 1.5 x 10^{-6} M BA. BA and 2iP levels above 5 x 10^{-6} M suppressed yields.

C. sunki (polyembryonic). 2iP showed no toxicity; the promotion by the 5 x 10^{-5} M concentration was statistically significant. BA showed slight but significant promotion at 1.5 x 10^{-6} M; higher levels were progressively repressive.

C. macrophylla (polyembryonic). This species also displayed no response to cytokinin addenda.

C. reticulata 'Cleopatra' (polyembryonic). Pseudobulbil development of this mandarin cultivar was generally very slow and response to cytokinin was not marked. Nevertheless, the slight improvement by a 5×10^{-6} M supplement of BA was significant. 2iP and all other levels of BA were ineffective.

C. reticulata 'Ponkan' (polyembryonic). The other mandarin cultivar displayed more vigorous pseudobulbil development and significant enhancement by 1.5 and 5×10^{-6} M concentrations of BA; concentrations above 5×10^{-6} were toxic. No significant effects were observed from 2iP.

C. grandis 'Ma Dou Bai You' (monoembryonic). This monoembryonic cultivar was promoted by a 2iP supplement of 1.5×10^{-5} M and the promotion improved by an additional supplement of 5×10^{-7} M NAA. BA showed no promotion, but slight suppression of pseudobulbils, particularly when combined with NAA. Rooting was stimulated by the auxin.

C. grandis 'Ma Dou Wen Dan' (monoembryonic). This shaddock was considerably more prolific than 'Ma Dou Bai You'. Also, NAA suppressed its pseudobulbil proliferation. Both cytokinins stimulated pseudobulbils at the lowest level, 1.5×10^{-6} M. BA was toxic in concentrations 1.5×10^{-5} and higher and caused browning of tissues, whereas 2iP showed no toxicity. NAA also stimulated rooting of this cultivar.

Discussion

The investigation disclosed that sustained proliferation and vigor of pseudobulbils, intermediary structures in citrus nucellar embryony in vitro (Rangaswamy, 1961), usually required a cytokinin, the kind and concentration of which varied according to species and cultivar. These species and cultivars responded more favorably to BA, and the optima were: *C. aurantium, C. jambhiri* and *C. reticulata* 'Ponkan', and *C. sinensis,* 1.5×10^{-6} M ; and *C. reticulata 'Cleopatra'*, 5×10^{-6} M. These showed superior effects by 2iP, and the optima were: *C. grandis* 'Ma Dou Wen Dan', 1.5×10^{-6} ; *C. grandis* 'Ma Dou Bai You', 1.5×10^{-5}; and *C. sunki,* 5×10^{-5} M. *C. limonia* and *C. macrophylla* required no cytokinin supplements. Pseudobulbil proliferation of the monoembryonic, *C. grandis* 'Ma Dou Bai You', but not 'Ma Dou Wen Dan', was further promoted by a 5×10^{-7} M addition of NAA. By using media with specified levels of cytokinin and auxin supplements, stock cultures of pseudobulbils of many of the species and cultivars have now been maintained without loss of vigor for over 5 years.

Development of plants from pseudobulbils has thus far resulted when pseudobulbils were left unsubcultured for longer periods. A multitude of other supplements, including galactose and GA_3, showed no effects on embryo emergence. Similarly, higher (37°C) and lower (15-17°C) incubation temperatures for varying periods were ineffective.

In many investigations of citrus nucellus culture, the pseudobulbils have been erroneously identified as embryos or embryoids and proembryos, e.g., Kochba *et al.,* 1972; Mitra and Chaturvedi, 1972; Rangan *et al.,* 1968; and Tisserat and Murashige ,1977abc). This was not unexpected, inasmuch as psedobulbils appear structurally, particularly during their early developmental stages, as embryos. They emerge as globules on surfaces of existing pseudobulbils, thus, the observation "budding"; elongate and eventually broaden into growths that are reminiscent of cotyledons and leaves. The precise origin in these structures and sequence of organogenetic steps in embryo development remains to be elucidated. Furthermore, the occurrence of pseudobulbils in nucellar embryogeny in vivo needs confirmation.

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Literature Cited

Button, J. and C. H. Bornman. 1971. Development of nucellar plants from unpollinated and unfertilised ovules of the Washington navel orange in vitro. J. S. Afr. Bot. **37:** 127-134.

Kochba, J., P. Spiegel-Roy, and H. Safran. 1972. Adventive plants from ovules and nucelli in *Citrus.* Planta **106:** 237-245.

Mitra, G. C. and H. C. Chaturvedi. 1972. Embryoids and complete plants from unpollinated ovaries and from ovules of in vivo-grown emasculated flower buds of *Citrus* spp. Bull. Torrey Bot. Club **99:** 184-189.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol.

Plant. **15:** 473-497.

Rangan, T. S., T. Murashige, and W. P. Bitters. 1968. In vitro initiation of initiation of nucellar embryos in monoembryonic *Citrus*. HortSci. **3:** 226-227.

Rangaswamy, N. S. 1961. Experimental studies on female reproductive structures of *Citrus microcarpa* Bunge. Phytomorph. **11:** 109-127.

Sabharwal, P. S. 1963. In vitro culture of ovules, nucelli and embryos of *Citrus reticulata* Blanco var. *Nagpuri*. *In* P. Maheshwari and N. S. Rangaswamy, eds., Plant Tissue and Organ Culture -- A Symposium. Intl. Soc. Plant Morph., Delhi, pp. 265-274.

Snedecor, G. W. 1946. Statistical Methods, 4th edn. Iowa State College, Ames.

Tisserat, B. and T. Murashige. 1977a. Effects of ethephon, ethylene, and 2,4-dichlorophenoxyacetic acid on asexual embryogenesis in vitro by some plant growth regulators in vitro. Plant Physiol. **60:** 437-439.

Tisserat, B. and T. Murashige. 1977b. Repression of asexual embryogenesis in vitro by some plant growth regulators. In Vitro **13:** 799-805.

Tisserat, B. and T. Murashige. 1977c. Probable identity of substances in citrus that repress asexual embryogenesis. In Vitro **13:** 785-789.

荷爾蒙對柑橘珠心組織假胚體分化之影響

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柑橘珠心組織培養時，培養基中加入 cytokinin 可導致假胚體之形成，此一球狀突起爲不定胚產生前之中間分化體，於試管培養時可見之。培養基中加入 cytokinin 之種類及濃度因橘種而異。椪柑、柳橙、酸橙、粗皮樟檬須加入 1.5×10^{-6} M BA，美女桔 5×10^{-6} M BA，酸桔 5×10^{-5} M 2iP，單胚柑橘之麻豆白柚須 1.5×10^{-5} M 2iP，麻豆文旦 1.5×10^{-6} M 2iP，可使假胚體繼代分化繁殖，黎檬則無須任何 cytokinin 亦可分化假胚體，培養基中若加入 NAA 5×10^{-7} M 有增進麻豆白柚假胚體分化之效果。