Wieser[11] also contends that the haemolymph protein compositions in a given species are affected by its ecological position. According to him, the relative haemocyanin content of the haemolymph tends to become more stable as the species acquires more terrestrial habitat. However, this hypothesis needs further verification.

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MORPHOGENESIS IN STEM-CALLUS TISSUE OF *CITRUS GRANDIS* IN LONG-TERM CULTURES—A BIOCHEMICAL ANALYSIS

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ABSTRACT

Stem-callus tissue of *C. grandis,* in long-term cultures, differentiated into two types of tissues : type-A, which was compact-nodular and slow growing and type-B, which was friable-spongy and fast growing. In a medium containing 0·25 mg/l BAP + 0·1 mg/l NAA + 500 mg/l ME, the type-A tissue produced numerous shoot-buds and shoots, whereas the type-B tissue did not. The two types of tissues also differed in respect of their nitrogen, protein, free amino acid and sugar contents.

INTRODUCTION

THERE are several reports[1–3] of the gradual loss of the regenerative capacity of plant callus tissues grown *in vitro* for a long time. Some workers[4–7] have studied the changing cytological conditions in the tissue during its prolonged culture and the correlated loss of its regenerative capacity. However, since callus tissue of varied ploidy including haploidy are known to differentiate organs and plantlets[8–10], and those with abnormal polyploid chromosome numbers, to form abnormal shoots[6], it appears that besides cytological alterations, some biochemical changes in the tissue during its prolonged culture may also be involved in the phenomenon of the loss of its morphogenetic potentiality. It seems that the latter aspect has not been studied so far, though some analyses of free amino acids in *in vitro*-grown tissues have been made[11–12]. In the present investigation, certain biochemical changes in long-term culture have been studied with a view to find out any correlation between the metabolic changes in the tissue and the loss of its regenerative capacity.

EXPERIMENTAL PROCEDURE

Composition (in mg/l) of MS medium, a variant of Murashige and Skoog's medium[13], where it differed from the litter, was : 150 NH_4NO_3, 1500 KNO_3, 400 $CaCl_2$, 150 KH_2PO_4, 360 $MgSO_4$. $7H_2O$, 10 thiamine-HCl, 2·5 pyridoxine-HCl, 2·5 nicotinic acid, 0·1 folic acid, 0·1 riboflavin, 0·1 biotin, 5 ascorbic acid, 50,000 sucrose and 7000 agar. Sterilization procedure and other cultural conditions were as reported earlier[14]. Stem-callus tissue of *C. grandis* was maintained in MS medium

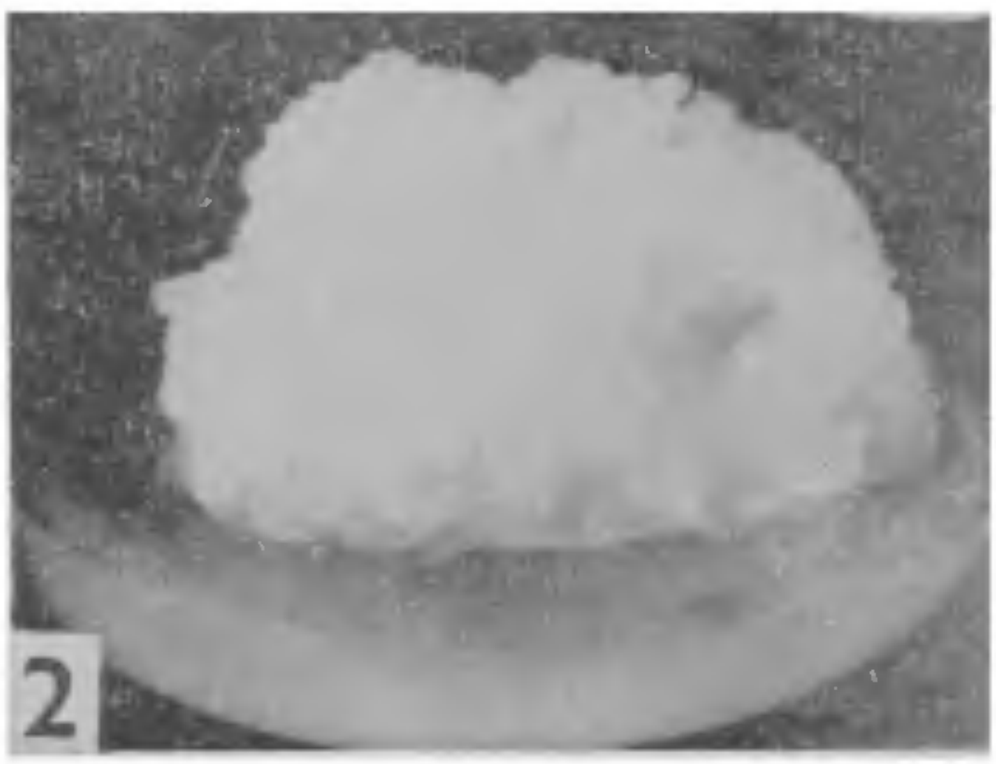
supplemented with 0·25 mg/l kinetin (Kn), 0·5 mg/l α-naphthaleneacetic acid (NAA) and 0·25 mg/l 2, 4-dichlorophenoxyacetic acid (2, 4-D) –MS–1 medium—for 2½ years and later in the same medium but devoid of Kn–MS–1*a* medium.

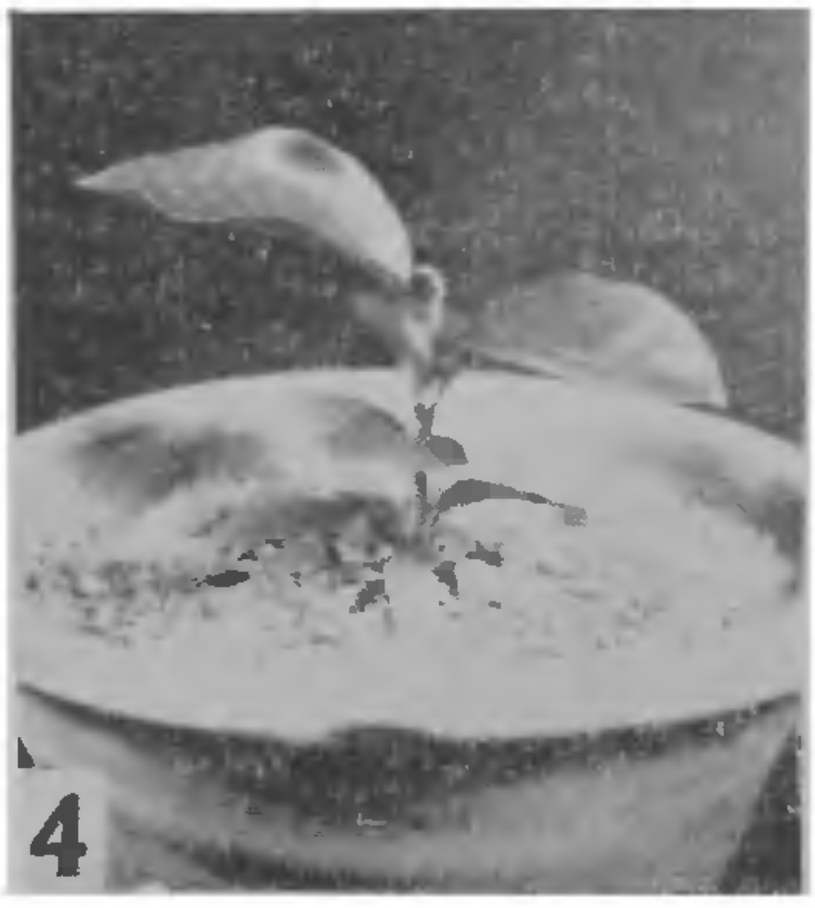
For organogenesis, the callus-tissue was cultured in MS–1*b* medium having half the concentrations of thiamine-HCl, pyridoxine-HCl and nicotinic acid, than present in MS medium and supplements of 0·25 mg/l 6-benzylaminopurine (BAP), 0·1 mg/l NAA and 500 mg/l malt extract (ME).

The callus tissue was analysed for quantitative determination of its nitrogen, protein, free amino acid and sugar contents. The nitrogen percentage of the callus tissue was determined by using modified Kjeldahl method[15–16] and the protein percentage was obtained by multiplying the former by 6·25. The quantitative determination of free amino acids of the callus tissue was made by paper chromatography following the procedure reported elsewhere[17]. The sugar content of the callus tissue was estimated after the method of Dubois *et al.*[18].

Experimental Results

Stem-callus tissue of C. *grandis* was compact-nodular and greenish-white in colour—named as Type-A tissue (Fig. 1). After its repeated sub-culturing for 2–2½ years in MS–1 medium, the Type-A tissue in certain cultures produced a friable-spongy tissue of pale-white colour. The friable-spongy tissue was isolated and grown in separate cultures and named as Type-B tissue (Fig. 2). The Type-B tissue showed profuse growth in MS–1*a* medium, which was far greater than that of the Type-A tissue in the same medium (Table I).





Figs. 1–4. Cultures of *Citrus grandis*. Fig. 1. Stem-callus tissue Type-A (× 0·99). Fig. 2. Stem-callus tissue Type-B (× 1·11). Fig. 3. Differentiation of shoots from stem -callus tissue Type-A (× 1·18). Fig. 4. A tissue-regenerated plant in potted soil (× 0·60).

TABLE I

Comparative growth in vitro *of two types of stem-callus tissues of* C. grandis *in MS–1*a *medium*

Stem-callus tissue	Wt. of inoculum*		Growth of explant*					
			10 days		20 days		30 days	
	Fr. wt. (gm)	Dry wt. (mg)	Fr. wt. (gm)	Dry wt. (mg)	Fr. wt. (gm)	Dry wt. (mg)	Fr. wt. (gm)	Dry. wt. (mg)
Type–A	0·4	60·16	0·4	68·86	0·6	77·84	0·8	125·72
Type–B	0·5	19	0·95	41	4·3	196·63	9·9	537·5

* Average of 5 replicate cultures.

In MS–*1b* medium, 2 to 2½-year-old Type-A tissue produced a large number of green shoot-buds which all developed into shoots (Fig. 3). It took about 60 days for shoot-buds to be visible on the callus surface and in the following 30 to 40 days they developed into shoots. Roots were not produced by the differentiating callus cultures. However, isolated tissue-regenerated shoots could easily be rooted in a different medium and were made to develop as complete plants in potted soil (Fig. 4). On the contrary, the Type-B tissue did not form any shoot-buds in the same medium though it became a bit less friable. Also, roots were not produced by the Type-B tissue. Same results were obtained by repeating the experiment for several times.

Stem-callus tissue : Type-A and Type-B not only showed differences in their morphology, growth rate and morphogenic potentiality, but also in their biochemical make-up in respect of nitrogen, protein, free amino acid and sugar contents, as presented in Table II. Concentrations of the chemical constituents assayed, *viz.*, nitrogen, protein, free amino acids and sugars were considerably less in Type-B tissue than in Type-A tissue. With respect to free amino acid content of the two types of tissues, they differed not only quantitatively, but also qualitatively, *i.e.*, fewer amino acids were present in the Type-B tissue. In all, 11 amino acids were present in Type-A tissue, whereas only 7 in the Type-B tissue. L-Glutamic acid, L-proline, L-serine and L-tyrosine were not present in the Type-B tissue. Asparagine, L-glutamic acid and glycine were the prominent amino acids in the Type-A tissue, whereas L-arginine, glycine and L-tryptophane in the Type-B tissue. Amongst the common amino acids present in both types of tissues, the decrease in their concentrations in the Type-B tissue with respect to Type-A tissue was most pronounced with L-asparagine, followed by L-threonine, L-aspartic acid, glycine, L-tryptophane,

TABLE II

Quantitative analysis of two types of stem-callus tissue of C. grandis *for their nitrogen, protein, free amino acid* and sugar* contents*

Chemical constituents		Stem-callus tissue	
		Type-A	Type-B
1. Nitrogen		1·337 %	0·5954%
2. Protein		8·356 %	3·722 %
3. Amino acids	L-Alanine	16·6	9·6
	L-Arginine	18·7	16·4
	L-Asparagine	45·8	10·7
	L-Aspartic acid	32·0	12·0
	Glycine	38·2	14·7
	L-Glutamic acid	42·0	..
	L-Proline	16·0	..
	L-Serine	20·5	..
	L-Threonine	25·0	8·02
	L-Tryptophane	31·6	14·1
	L-Tyrosine	12·2	..
4. Sugars	D-Glucose	198·0	62·3
	Sucrose	99·0	50·4
	Fructose	81·0	39·0

* Quantity in μg/100 mg fr. wt of callus tissue; data based on three chromatographs of each sample.

L-alanine and L-arginine. Amongst sugars, D-glucose was the most prominent in both the types of tissues, followed by sucrose and fructose.

Discussion and Conclusion

During prolonged culturing, the plant tissues are known to undergo many kinds of changes and not infrequently new strains have been obtained differing in one or more such characteristics as texture, compactness, colour, growth rate, growth requirements, and morphogenetic potentiality. To cite a few examples: in cultures of carrot, strains with compact type of growth resulted from highly friable parent strain[19], callus strains of *Trichocereus* differed in growth rates and texture[20], whereas those of *Melilotus* and *Opuntia* calli in pigmentation[21]. Similarly, in the present case, during prolonged cultures of stem-callus tissue of *C. grandis*, a new type of tissue named Type-B, which was friable-spongy, pale-white and fast growing, resulted from parent Type-A tissue characterized by being compact-nodular, greenish-white and slow growing.

In contrast with the situation obtained in long-term cultures of pea root-callus, where the callus strain of hard texture and reduced growth rate, originated from friable and comparatively fast growing parent tissue, lost the organogenetic capacity[3], the friable and fast growing type of citrus tissue, *i.e.*, Type-B derived from compact-nodular and slow growing tissue, did not form organs. This tissue (Type-B) lacking regenerative potentiality not only had strikingly less concentrations of free amino acids, nitrogen, protein and sugars than that found in the potentially differentiating tissue, *i.e.*, Type-A, but also lacked certain amino acids altogether, *viz.*, L-glutamic acid, L-proline, L-serine and L-tyrosine. Thus in the present case, the loss of regenerative potentiality seems to be linked with fast growth and certain concurrent changes in the biochemical composition of the tissue.

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