Enzymes of sucrose metabolism in groundnut (Arachis hypogaea) callus and developing cotyledons

S K Yadav, T Radhakrishnan & J B Misra*

National Research Centre for Groundnut, P O Box No. 5, Junagadh 362 001, India

Groundnut (A. hypogaea L.) calli were cultured on three MS-media differing in carbon sources namely fructose (callus-F), glucose (callus-G) and sucrose (callus-S). Developing cotyledons of field grown plants and three calli developed in vitro were analysed for glucose, fructose and sucrose contents and activities of sucrose phosphate synthase (SPS), sucrose synthase (SS) and invertase. Sucrose was 81, 91, 125, and 82 μmole/g fr wt in cotyledons, callus-F, callus-G, and callus-S, respectively. Glucose and fructose contents varied widely. Callus-F was characterised by high sucrose and fructose contents, callus-G by high sucrose and glucose contents, and callus-S by high sucrose and low glucose contents. Compared to SS, both SPS and invertase were insignificant in callus-F and callus-G. On the basis of relative abundance of SS and SPS in three calli, the presence of sucrose in callus-F and callus-G was ascribed to de novo synthesis of sucrose by SS.

It is currently believed that synthesis of sucrose, the main form of photosynthetic carbon export, is catalysed by sucrose phosphate synthase (SPS) while cleavage of sucrose at the site of its utilisation is catalysed by sucrose synthase (SS)[1,2]. In most of the plants, both the enzymes have been detected regardless of whether plant tissues were concerned with export or utilisation of sucrose. However, SS has been found to be several fold higher in tissues that are net importers of carbon (sink)[1-4] while the opposite is true for tissues that are net exporter of carbon(source)[5-7]

Moreover, because the developing natural sinks import ready-made sucrose from the source, they have little requirement of enzymes of sucrose biosynthesis. Hence, the developing natural sinks are characterised by having either a very low levels of SPS (compared to that of SS)[3,7,8] or lack of it[1].

Of late, the activity of SS has been shown to be correlated with sweetening (accumulation of sucrose) of sinks in fruits of cucumber[9], pepino[10] and muskmelon[11]. More recently it has been shown that SS catalyzes reversible reaction in developing potato tubers and some other plant tissues[12].

Conclusions drawn on the basis of correlations between the levels of SS or SPS and the pool-sizes of sucrose in developing sinks[9,11,13-15] suffer from the

drawback that in such cases it is difficult to ascertain the relative contribution of import and de novo production of sucrose to the sucrose pool. Callus in a culture medium is analogous to natural sink of plant as it also draws ready-made carbon skeletons from an external source, the culture medium. Unlike natural sinks, however, for a growing callus, the nature of sugar being imported can easily be manipulated by using sugars other than sucrose in the medium. And this novel approach to studying the sucrose metabolism in groundnut was adopted in the present experiment..

There is limited information on relative activities of SS and SPS in a developing callus and corresponding natural sink of groundnut (A. hypogaea L.). Thus it was of interest to compare the enzymes of sucrose metabolism between calli growing on three media differing in carbon sources and the natural sink of groundnut plant i.e. developing cotyledons.

Materials and Methods

Callus culture—Immature leaves (3-5 mm) of in vitro germinated 7-day old seedlings of A. hypogaea L. cultivar J 11 were used as explants. MS medium[16] with vitamins of B5 medium[17] was used to culture the explants on three different sources of carbon namely fructose (0.2 mole/l), glucose (0.2 mole/l) and sucrose (0.1 mole/l). Growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA; 0.1 mg/l each) were also incorporated in the medium. Calli were harvested after 20 days in their

*Correspondent author

rapid growth phase (stage-I) and subsequently after 45 days in their maturation phase (stage-II). Extraneous material, if any, adhering to the tissue was fully removed with help of needles followed by washing with water.

Developing cotyledons—Developing cotyledons were collected from bulk crop raised at the research station with standard cultural practices. Milky cotyledons, half-filled pods, (stage-I) and well-developed cotyledons, almost fully-filled pods, (stage-II were used for this study.

Extraction of enzymes—Enzymes were extracted by the method outlined by Huber[6]. Tissue was ground in a glass mortar with acid washed sand and 50 mM HEPES-NaOH buffer (pH 7.5) containing 2-mercaptoethanol (30 mM), PVPP (1%), EDTA (1 mM) and MgCl$_2$ (5 mM). Ratio (w/v) of homogenizing buffer to tissue was 1:3 for calli and 1:4 for developing cotyledons. The slurry was passed through 2 layers of muslin and then centrifuged at 14,000 g for 30 min at 4°C. The supernatant was passed through a column of Sephadex G-25 equilibrated with 50 mM Tris-HCl (pH 7.5) containing MgCl$_2$ (5 mM) and 2-mercaptoethanol (5 mM). The gel filtrate was used as enzyme source. All operations were carried out at 2°-4°C.

Assay of enzymes—Assay mixture (0.4 ml) for SPS (UDP-glucose; D-fructose-6-phosphate-2-glucosyl-transferase, EC 2.4.1.14) contained Tris-HCl (100 mM; pH 7.5), NaF (1 mM), UDP-glucose (8 mM), fructose-6-phosphate (8 mM), MgCl$_2$ (12 mM) and enzyme (0.15 ml). Reaction was started by addition of UDP-glucose. Assay mixture for SS (UDP-glucose: D-fructose-2-glucosyltransferase, EC 2.4.1.13) was identical except that fructose was substituted for fructose-6-phosphate and that NaF was deleted. The assay mixtures were incubated at 30°C for 10 min and then terminated by the addition of NaOH (0.1 ml of 2.5 mole/l) and heating in a boiling water bath for 10 min to destroy unreacted fructose-6-phosphate or fructose. Sucrose phosphate or sucrose formed as a result of reaction was measured by the method of Roe as described by Ashwell[18]. One unit of enzyme was defined as nmole of substrate transformed per second (nkat) under assay conditions. Protein in gel filtrate was determined by the method of Lowry *et al*[19].

Invertase (β-D-fructofuranoside fructohydrolase, EC 3.2.1.26) was assayed as described earlier[13] with slight

modifications. The assay mixture (0.5 ml) contained sucrose (300 mM), citrate-phosphate (60 mM, pH 5) for acid invertase and sucrose (mM) HEPES-NaOH (60 mM, pH 7.5) for neutral or alkaline invertase and enzyme (0.2 ml). The assay mixture was incubated at 30°C for 10 min and reducing sugars liberated were determined colorimetrically as described by Ashwell[18].

Extraction and determination of sugars—Tissue (1 g) was cut into find pieces and extracted twice with 80% ethanol (5 ml) under reflux. After removal of pigments with CHCl$_3$, the extract was concentrated to 1 ml by evaporating ethanol. Concentrated extract was used for separating sugars on a 'Shimadzu' HPLC LC-9A system which consisted of a refractive index detector [model RID-6A] CLC-NH$_2$ (4.6×250 mm) column and C-R6A injector. Concentrated extract (20 μl) was injected and sugars were eluted with acetonitrile:water (75:25) at 1 ml per min. Identification and quantification of sugars was done by comparison with standard chromatogram.

Results and Discussion

Sugar composition of developing cotyledon and calli are shown in Table 1. Compared to glucose and fructose, sucrose was more in the developing cotyledons at both stage-I and -II, although its concentration at stage-I was four times that of stage-II. While fructose content was lower, glucose content was much higher at stage-I than stage-II. At stage-I of developing cotyledon, sucrose content was highest, followed by almost equal quantities of fructose and glucose. However, at stage-II, sucrose concentration was followed by fructose and glucose, glucose concentration being significantly lower than fructose concentration.

At stage-I, sucrose was present in the highest

Table 1—Soluble sugars of groundnut cotyledon and calli

[Values are mean of 3 determinations]

Tissue		Sugars (μmole g^{-1} fr wt)		
		Sucrose	Fructose	Glucose
Cotyledon	I	80.78	7.67	7.70
	II	20.11	9.01	3.67
Callus-F	I	91.30	31.41	0.15
	II	3.77	3.06	(n.d.)
Callus-G	I	125.25	0.33	12.42
	II	1.90	(n.d.)	2.50
Callus-S	I	81.84	0.22	3.47
	II	4.56	(n.d.)	(n.d.)

n.d.—not detectable; I—Stage I; and II—Stage II

Table 2—Activities of SS, SPS, and invertase in developing cotyledon and calli of grounnut

[Values are mean of 3 determinations]

Tissue		SS	SPS	Invertase pH 5.0	Invertase pH 7.5	Extractable protein (mg g⁻¹ fr wt)
Cotyledon	I	59.2 (135)	0.46 (1.1)	0.76 (1.74)	1.98 (4.53)	26.25
	II	42.2 (240)	1.95 (11.1)	—	—	10.52
Callus-F	I	16.7 (305)	0.31 (5.68)	0.38 (7.03)	4.53 (82)	3.28
	II	3.9 (46.4)	(n.d)	—	—	5.04
Callus-G	I	10.8 (197)	0.21 (3.83)	0.21 (3.90)	2.90 (52.8)	3.29
	II	3.9 (31.1)	(n.d.)	—	—	7.63
Callus-S	I	5.6 (221)	0.34 (13.3)	0.17 (6.7)	1.24 (48.6)	1.53
	II	2.9 (25.5)	(n.d.)	—	—	6.81

Table header units: Activity (nkat g⁻¹ fr wt)

Parenthesis indicate activity nkat mg⁻¹ protein min⁻¹; n.d.—not detectable; I—stage I; and II—stage II

quantity in all the three calli. Fructose was present at the second highest levels in callus-F and glucose in callus-G.

In the developing cotyledons, at stage-I, SS was about 128, 78, and 30 times that of SPS, acid invertase and alkaline invertase, respectively (Table 2). At stage-II, however, SS was 22 times that of SPS. Activity of invertase was not determined at stage-II.

Whereas all the four enzyme activities namely SPS, SS, acid invertase and alkaline invertase could be easily determined in all the calli at stage-I, the activity of SPS could not be detected in any of the calli at stage-II by the protocols employed. In callus-F, SS was 54, 44, and 3.7 times that of SPS, acid invertase and alkaline invertase, respectively. Almost a similar ratio was found in callus-G, in which SS was 51 times that both SPS and acid invertase and 3.7 times that of alkaline invertase. In callus-S, however, the differences in the levels of SS and SPS enzymes were rather narrow and SS was 16.5, 33, and 4.5 times that of SPS, acid invertase and alkaline invertase, respectively.

Low level of sugar and activities of enzymes in the three calli at their maturation phase (stage-II) was ascribed to the reduction in the rates of metabolism due to senescence.

Cells of higher plants are endowed with the ability of producing any two of glucose, fructose and sucrose from the remaining third sugar. Since the calli are quite analogous to a natural sink in that they also receive ready-made sucrose from the supporting medium, in the present experiment, absence of SPS (or its presence in negligible quantities in relation to SS) in the three calli was in conformity with their being a tissue analogous to sink.

Accepting exclusive roles in the direction of synthesis and degradation for SPS and SS, respectively, and also considering sucrose to be ubiquitous in plant cells, in the present experiment it was expected that—(i) callus-F and callus-G, which both could import a monosaccharide from the medium would have little SS but appreciable levels of SPS to produce sucrose; and (ii) callus-S, which could import ready-made sucrose would have little SPS and high SS to degrade sucrose for its utilisation.

But the results of the experiment did not conform to expectations for callus-F and callus-G. Since callus-F and callus-G could not import ready-made sucrose, its presence in these two calli was ascribed entirely to de novo synthesis. Compared to SS, activities of both SPS and invertases were not found appreciably high in callus-F and callus-G. Moreover, because both callus-F and callus-G could take up carbon skeleton in the form of monosaccharide from the supporting medium, the presence of SS in these two calli and with even higher activities than that of callus-S could not be explained on the basis of the requirement of cleavage of sucrose. Hence the synthesis of sucrose in callus-F and callus-G was ascribed to SS which not only was present in large quantities but also had no other role to perform in these two calli. It was, thus, concluded that SS, which is a constitutive enzyme of plant sink

tissues, can be invoked for the synthesis of sucrose also in case of need.

A degradative role for SS has been accepted on the basis of its pattern of distribution in various source and sink tissues and also its kinetic properties. As a matter of fact, the reaction catalysed by SS has been shown to be an easily reversible one *in vitro*[20], so much so the enzyme is generally assayed *in vitro* by a majority of researchers in the direction of synthesis only[1,11,13,14]. Recently, it has been shown that SS catalyses a reversible reaction *in vivo* too in developing potato tubers and other plant tissues[12]. The results also support the hypothesis that like some natural sinks, the callus growing on a fructose or glucose medium may use reversibility of SS for biosynthesis of sucrose.

References

1 Giaquinta R, *Plant Physiol,* 61 (1978) 380.

2 Echeverria E & Humphreys T, *Phytochemistry,* 23 (1984) 2173.

3 Hawker J S, *Phytochemistry,* 10 (1971) 2313.

4 Pressey R, *Plant Physiol,* 44 (1969) 759.

5 Bird I F, Cornelius M J, Keys A J & Whittingham C P, *Phytochemistry,* 13 (1974) 59.

6 Huber S C, *Z Pflanzenphysiol,* 102 (1981) 443.

7 Misra J B, Daniel E V & Premchand, *Plant Physiol Biochem,* 32 (1993) 131.

8 Fieuw S & Willenbrink J, *J Plant Physiol,* 131 (1987) 153.

9 Schaffer A A, Aloni B & Fogelman E, *Phytochemistry,* 26 (1987) 1883.

10 Schaffer A A. Rylski I & Fogelman M, *Phytochemistry,* 28, (1989) 737.

11 McCollum T G, Huber D J & Cantliffe D J, *J Am Soc Hort Sci,* 113 (1988) 399.

12 Geigenberger P & Stitt M, *Planta,* 189 (1993) 329.

13 Stommel J R, *Plant Physiol,* 99 (1992) 324.

14 Miron D & Schaffer A, *Plant Physiol,* 95 (1991) 623.

15 Dali N, Michaud D & Yelle S, *Plant Physiol,* 99 (1992) 434.

16 Murashige T & Skoog F, *Physiol Plant,* 15 (1962) 473.

17 Gamborg O L, Miller R A & Ojima K, *Exp Cell Res,* 50 (1968) 151.

18 Ashwell G, in *Methods in enzymology,* Vol. 3, edited by S P Colowick & N O Kaplan (Academic Press, New York) 1957, 73.

19 Lowry O H, Rosebrough N J, Farr A L & Randall R J, *J Biol Chem,* 193 (1951) 265.

20 Akazawa T & Okamoto K, in *Biochemistry of plants* , Vol. 3, edited by P K Stumpf and E E Conn (Academic Press, New York) 1980, 199.