

Trehalose Toxicity in *Cuscuta reflexa*¹

SUCROSE CONTENT DECREASES IN SHOOT TIPS UPON TREHALOSE FEEDING

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ABSTRACT

Trehalose, an α,α -diglucoside, induced a rapid blackening and death of shoot tips of *Cuscuta reflexa* (dodder) cultured *in vitro*. The onset of toxic symptom was delayed if any of the several sugars which support the *in vitro* growth of *Cuscuta* was supplied with trehalose. The rate of trehalose uptake or its accumulation in the tissue was not affected by sugar co-feeding. The levels of total and reducing sugars declined appreciably in the trehalose-fed shoot tip explants compared to control tissue cultured in absence of a carbon source. This was not due to an increased rate of respiration of the trehalose-treated tissue. In shoot tips cultured in presence of both trehalose and sucrose, the decline in total and reducing sugars was curtailed. There was a marked fall in the level of sucrose; and invertase activity was higher in trehalose-fed shoot tips. The incorporation of label from [¹⁴C]glucose into sucrose in the shoot tip explant was reduced as early as 12 h of trehalose feeding. The results suggest that increased utilization of sucrose as well as an inhibition of its synthesis contribute to the drastic fall in the sucrose content upon trehalose feeding.

In a previous paper (13) we reported that the nonreducing disaccharide, α,α -trehalose, induced a rapid blackening of excised shoot tips of *Cuscuta reflexa* (dodder) cultured *in vitro*. This effect of trehalose was specific insofar as several mono-, di-, and trisaccharides tested were not toxic and these generally supported good growth of shoot tips. The addition of a utilizable sugar delayed the blackening of the shoot tips. This observation suggested that trehalose may interfere in some metabolic pathway involving sugars. With a view to understanding the mechanism of trehalose toxicity in *Cuscuta*, we have investigated some aspects of carbohydrate metabolism in shoot tips following trehalose feeding. We have found a decrease in the contents of carbohydrates and, in particular, of sucrose upon trehalose feeding.

MATERIALS AND METHODS

In Vitro Culture of *Cuscuta*. The culture medium (6) was the same as that used earlier except that medium lacking sugar is designated as BM.³ Shoot tips of *Cuscuta* were cultured by two methods. Method I, unless mentioned otherwise, is the same as described earlier (6); 2.5-cm long shoot tips were cut from primed and surface-sterilized tissue and cultured aseptically. In method II, 3.5-cm long shoot tips were marked with indelible ink 2.5 cm below the apex and used without priming and surface-sterilization.

The tips were placed singly in tubes containing 0.3 ml medium which was changed daily to minimize microbial growth. At the end of the growth period the shoot tips were cut at the mark and the terminal portion assayed for carbohydrates.

Extraction and Estimation of Starch and Sugars. Starch was extracted from the tissue by the method of ap Rees *et al.* (1). An aliquot of the extract was incubated for 3 h at 50°C with amyloglucosidase (0.75 unit) and α -amylase (65 units) in 50 mM sodium acetate buffer (pH 5.6) in a total volume of 1 ml. Glucose formed was estimated by glucose oxidase-peroxidase method (2). Starch content was determined from a standard curve of glucose obtained by the hydrolysis of known amounts of starch by the same procedure.

Ethanol extracts rendered free of lipids, were estimated for total sugars by the method of Yemm and Willis (14) and for reducing sugars by the method of Somogyi (11). Trehalose was estimated using trehalase as described earlier (13). Sucrose in the neutral fraction (13) was estimated using invertase from *Candida utilis*. For this, an aliquot of the neutral fraction was incubated for 2 h at 40°C with invertase (28 units) in a total volume of 1 ml made up with 50 mM sodium acetate buffer (pH 5.5). The reaction was stopped by immersing the tubes in a boiling water bath and glucose formed was estimated by glucose oxidase-peroxidase method. Sucrose content was calculated by multiplying the glucose value by 1.9.

Measurement of Invertase Activity. Cell-free homogenate of shoot tips was prepared as described previously (13). Invertase was assayed by glucose oxidase-peroxidase method after hydrolysis of sucrose. The reaction mixture contained 0.2 ml 200 mM sucrose and a suitable volume of the homogenate in a total volume of 1 ml made up with 50 mM sodium acetate buffer (pH 5.6). The mixture was incubated for 20 min at 30°C and the reaction was stopped by placing the tubes in a boiling water bath for 2 min. Protein was estimated by the method of Potty (9).

Respiration Measurements. O₂ uptake was measured with an Aminco Warburg apparatus by standard procedures. Respiration was also studied by measuring the ¹⁴CO₂ released from [U-¹⁴C]glucose. Five-mm segments from 5 shoot tips were placed in the main compartment of the Warburg flask containing 2 ml BM. The center well contained 0.3 ml 10% KOH and 0.5 ml 5% TCA was placed in the side-arm. After the addition of 100 μ l [U-¹⁴C]glucose solution (about 650,000 cpm) the flasks were incubated for 2 h at 30°C on a metabolic shaker. Respiration was terminated by tipping TCA from the side-arm into the main compartment. After 20 min, radioactivity in KOH solution was measured by the method of Madsen (5).

Isotope Experiments. For study of trehalose uptake, shoot tips were cultured in tapered tubes containing 0.1 ml medium. [U-¹⁴C]trehalose was added to culture media. At 12-h intervals, radioactivity remaining in the culture medium was determined and the difference between the initial and final radioactivity was taken as a measure of [¹⁴C]trehalose taken up by the tissue.

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³ Abbreviation: BM, basal medium.

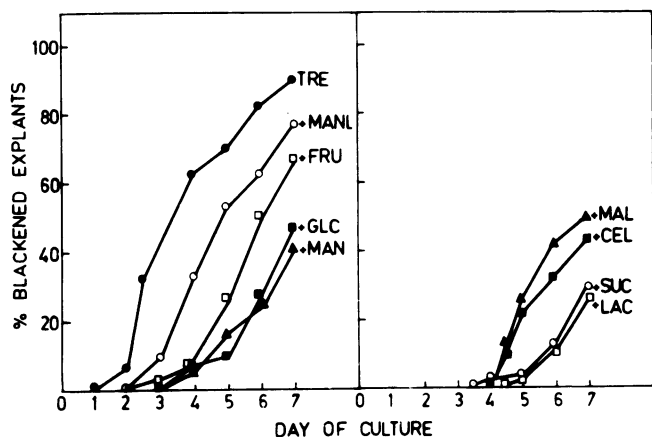


FIG. 1. Time-course of trehalose toxicity in *Cuscuta* shoot tips in the absence and in the presence of another sugar. The shoot tips were cultured by method I in medium containing 1% trehalose or 1% trehalose + 1% test sugar. Number of replicates in each treatment was 30. Abbreviations of sugars are as follows: TRE-trehalose, MANL-mannitol, FRU-fructose, GLC-glucose, MAN-mannose, MAL-maltose, CEL-cellobiose, SUC-sucrose and LAC-lactose.

For studying the fate of labeled sucrose, shoot tips were cultured aseptically in tapered tubes. About 241,000 cpm of [14 C]sucrose mixed with 1 μ mol unlabeled sucrose was added to 0.2 ml medium in each tube. At 12-h intervals, three shoot tips from each treatment were analyzed for radioactivity. Uptake of sucrose was determined as for trehalose.

Incorporation of [14 C]glucose into sucrose was studied as follows. Eight-cm long vines of *Cuscuta* were marked 2 cm below the apex and cultured for up to 60 h by method II. Vines were removed at 12 h intervals and cut at the mark and three shoot tips from identically treated vines were placed for 2 h at 30°C in a test tube (10 \times 1.3 cm) containing 0.1 ml BM and about 1,482,000 cpm of [14 C]glucose. At the end of the incubation period the shoot tips were used for analysis. Uptake of glucose was determined as for trehalose.

Measurement of Radioactivity. Solutions for radioactivity measurements were spotted on 1.8 \times 1.8 cm Whatman No. 3 paper and counted in 5 ml 0.5% PPO in toluene. Radioactivity was determined in a Beckman LS-100 scintillation counter.

Chemicals. Inorganic chemicals, solvents, sucrose, and glucose were of analytical grade. Amyloglucosidase (from *Rhizopus*), α -amylase (from *Bacillus subtilis*), glucose oxidase (from *Aspergillus niger*), invertase (from *Candida utilis*), peroxidase (Type II, from horseradish) and other biochemicals were bought from Sigma. [14 C]

[14 C]trehalose (540 mCi/mmol) was bought from the Radiochemical Centre, Amersham, England. [14 C]glucose (114 mCi/mmol) and [14 C]sucrose (10 mCi/mmol) were bought from Bhabha Atomic Research Centre, Trombay, India.

RESULTS

Antagonism of Sugars on Trehalose-Induced Blackening. In medium containing 2% trehalose, 50% shoot tips blackened after 3 days, whereas in 2% trehalose + 2% sucrose, blackening did not occur by that time. Figure 1 gives the results of an experiment in which the time-course of trehalose toxicity was studied in the presence of various sugars at 1%. All sugars tested delayed blackening. For example, in trehalose alone, 63% of the explants blackened on the 4th d, whereas in combination with another sugar, less than 5% of the explants had blackened at that time. Mannitol, which does not promote the *in vitro* growth of excised *Cuscuta* shoot tips (13), was, however, not very effective in delaying blackening. Thus, in the presence of a growth promotory (utilizable) sugar, the symptom of trehalose toxicity was delayed, although in 2 to 3 weeks blackening of almost all explants had occurred. The rate of [14 C]trehalose uptake by the shoot tips was approximately the same in the absence or the presence of sucrose up to 24 h and thereafter the rate of uptake of the label was, in fact, higher in the presence of sucrose. Trehalose accumulated within the tissue at the same rate in shoot tips cultured in the presence or in the absence of sucrose (Table I). Thus, the action of sucrose and possibly of the other sugars was within the tissue. Therefore, the effect of trehalose feeding on the carbohydrate content was investigated.

Changes in Carbohydrate Content. Shoot tips were cultured by method II. Initially, total sugars, reducing sugars, and starch comprised close to 1.9, 0.5, and 1.5%, respectively, of the fresh weight of the shoot tip. On day 1, the total sugar content increased in all treatments (Fig. 2). Thereafter, in shoot tips grown in BM or in BM + sucrose, the levels of total sugars remained nearly the same up to day 3. In contrast, the total sugar content in the trehalose-treated shoot tips decreased. By day 3, it was 43% of that in the BM-grown shoot tips. The value for the total sugar includes that for trehalose which accumulates in the shoot tips. If the trehalose content is deducted from the total sugar content the fall in the total sugars would appear more marked. The levels of reducing sugars also decreased markedly, being 66% of the initial level in trehalose-fed tissue on day 3 (Fig. 2B). Starch content in shoot tips grown in all three media diminished by 50% of the initial level by day 1 (Fig. 2C). By day 3, when blackening of trehalose-fed tissue had begun, the starch content of the shoot tips in all treatments had decreased further. The rapid utilization of

Table I. Effect of Sucrose Co-Feeding on the Levels of Total and Reducing Sugars and Trehalose in *Cuscuta* Shoot Tips Fed Trehalose

Shoot tips^a were cultured by method I in BM + 2% trehalose or BM + 2% trehalose + 2% sucrose. Five shoot tips were combined for sugar estimation. Values are means of three independent determinations.

Treatment	Day of Culture	Trehalose	Total Sugars	Total Sugars - Trehalose	Reducing Sugars
			$\mu\text{g/explant} \pm \text{SE}$		
BM + trehalose ^b	2	73 \pm 5	519 \pm 10	446	195 \pm 9
	4	121 \pm 12	413 \pm 33	292	121 \pm 19
	6	209 \pm 19	387 \pm 24	178	108 \pm 5
BM + trehalose + sucrose	2	77 \pm 11	609 \pm 40	532	210 \pm 23
	4	126 \pm 7	696 \pm 16	570	200 \pm 6
	6	230 \pm 23	647 \pm 36	417	186 \pm 12
	8	395 \pm 57	857 \pm 100	462	188 \pm 10

^a The initial content of total sugars and reducing sugars was 528 \pm 14 and 197 \pm 6 μg , respectively.

^b These explants had blackened on day 4; therefore explants were not analyzed after day 6.

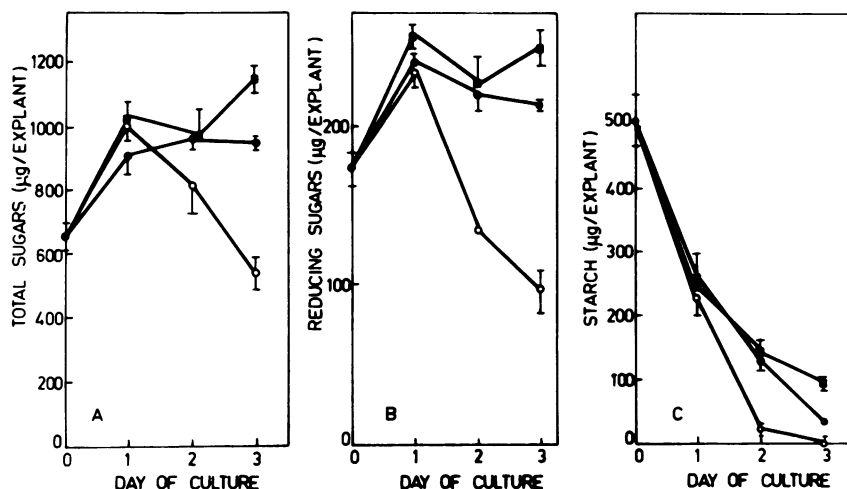


FIG. 2. The contents of total sugars (A), reducing sugars (B), and starch (C) in the shoot tips of *Cuscuta*. The shoot tips were cultured by method II in BM (●—●), BM + 2% sucrose (■—■), and BM + 2% trehalose (○—○). Five shoot tips were combined for carbohydrate estimation. Values are means of three independent measurements. Bars indicate SE.

starch in all treatments was apparently a response to excision—a phenomenon known in the cut tissues (4).

Whether sucrose co-feeding curtailed the trehalose-induced changes in the carbohydrate content was investigated. In shoot tips supplied with trehalose and sucrose, the contents of total sugars (minus trehalose) and reducing sugars remained almost constant up to day 6 (Table I). This was in contrast to shoot tips supplied with trehalose alone. However, even the shoot tips co-fed with sucrose had begun to blacken on day 6.

Respiration. Whether an increased respiratory rate contributed to the decrease in the contents of soluble sugars in the trehalose-fed shoot tips was investigated. Shoot tips were cultured in BM and in BM + 2% trehalose. Respiration of trehalose-fed shoot tips was not affected on day 1. However, after 2 days in trehalose-medium, their respiratory rate was 63% of the control (BM) tissue. At the onset of blackening (day 3), the respiratory rate of the treated shoot tips was 50% of the control.

Sucrose. The observed decline in soluble sugar content upon trehalose feeding prompted an investigation of the content of sucrose in the shoot tips. The initial (day 0) sucrose content of a 2.5-cm long shoot tip was 140 µg which amounts to about 0.5% of the fresh weight of the tissue. In Figure 3, the sucrose content is expressed as percentage of the initial value. In 4 d, in shoot tips cultured in BM and BM + 2% sucrose, sucrose content increased to 124 and 210%, respectively. On the other hand, in the explants cultured in BM + 2% trehalose, content of sucrose was 8%. In shoot tips cultured in BM + 2% trehalose + 2% sucrose it was 79%.

The rate of uptake of [¹⁴C]sucrose by the shoot tips in the absence or the presence of trehalose in the medium was linear and nearly the same up to 60 h (data not shown). To study the fate of [¹⁴C]sucrose taken in by the shoot tip, the ethanolic extracts freed of lipids were chromatographed as described earlier (13) and the radioactivity in sucrose was measured. In shoot tips cultured in BM, the label in sucrose increased linearly with time (Fig. 4). In contrast, in trehalose-fed shoot tips, label in sucrose did not increase although the rate of sucrose uptake was the same in both treatments. The difference in the utilization of sucrose was evident within 24 h of trehalose feeding.

The specific activity of invertase in shoot tips cultured in BM or BM + sucrose decreased to one-third of the initial (day 0) value within a day of culture and remained low till day 3. In trehalose-fed shoot tips, the specific activity of invertase initially decreased (day 1), but thereafter it increased. At the time of blackening (day 3), the specific activity of the enzyme was twice of that in control

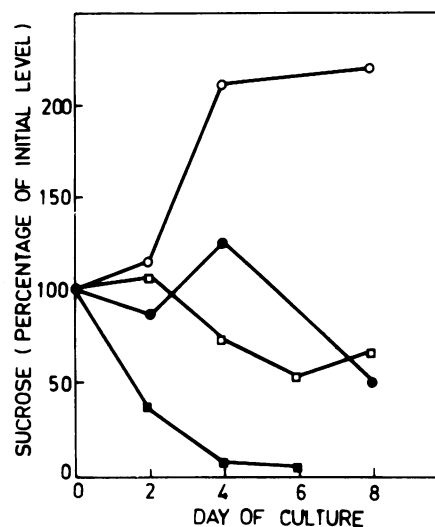


FIG. 3. Content of sucrose in the shoot tips of *Cuscuta*. Shoot tips were cultured by method I in BM (●—●), BM + 2% sucrose (○—○), BM + 2% trehalose (■—■), and BM + 2% trehalose + 2% sucrose (□—□). Five shoot tips were combined for sucrose estimation. Values represent means of three replicates.

tissue. The decrease in the levels of sucrose in the trehalose-fed explants was associated with the increase in invertase activity.

The incorporation of label from [¹⁴C]glucose into sucrose by the tissue was studied in an attempt to obtain more information about the low sucrose in shoot tips following trehalose feeding. The uptake of [¹⁴C]glucose by the shoot tip was affected to a small extent (about 10%) upon trehalose feeding. The incorporation of radioactivity in the neutral fraction was approximately 50% less in the shoot tips within 12 h of trehalose feeding compared to control (BM) shoot tips. The neutral extracts were chromatographed and as a representative example, the distribution of radioactivity in different sugars in the neutral fractions from 60 h grown explants is shown in Figure 5. In the control shoot tips, the peak radioactivity was in sucrose (Fig. 5A). In contrast, in the trehalose-fed shoot tips, the radioactivity in sucrose was very low (Fig. 5B). Incorporation of label from [¹⁴C]glucose into sucrose was reduced as early as 12 h in trehalose-fed shoot tips (Table II). The results showed that an inhibition of sucrose biosynthesis is an early biochemical symptom of trehalose toxicity.

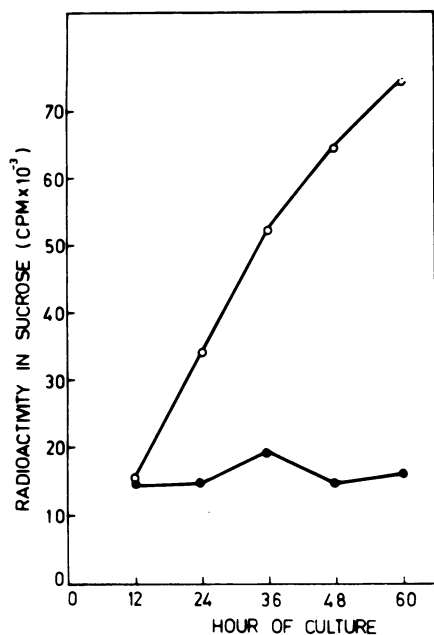


FIG. 4. Retention of exogenously fed [¹⁴C]sucrose in the shoot tips of *Cuscuta* cultured in the absence and in the presence of trehalose. Shoot tips were cultured by method I in 0.2 ml BM (○—○) or BM + 2% trehalose (●—●) containing [U-¹⁴C]sucrose (241,000 cpm). The aqueous fraction from the shoot tips was chromatographed and the distribution of radioactivity in the chromatogram was determined. Radioactivity in sucrose is expressed as cpm/3 explants.

Table II. Effect of Trehalose Feeding on the Incorporation of Label from [¹⁴C]Glucose into Sucrose

The shoot explants were grown by method II in BM or BM + 2% trehalose. At intervals of 12 h, 3 shoot tips (2 cm) from each treatment were fed with [¹⁴C]glucose for 2 h. The neutral fractions were chromatographed.

Period of Growth	Radioactivity in Sucrose		Radioactivity in Sucrose as % of Total [¹⁴ C]Glucose Uptake	
	BM	BM + Trehalose	BM	BM + Trehalose
<i>h</i>	<i>cpm/3 explants</i>			
12	6,601	1,865	3.68	1.14
24	7,286	1,199	3.94	0.68
36	9,002	2,174	5.68	1.04
48	ND ^a	1,739	ND	0.68
60	18,392	2,383	6.19	1.27

^a Not determined.

DISCUSSION

Because trehalose is a diglucoside and because glucose co-feeding effectively delayed toxicity symptoms, we may rule out toxicity as due to the accumulation of products of hydrolysis of trehalose. As reported earlier (13), *Cuscuta* has low trehalase activity. As a result, significant amount of trehalose taken in by the tissue would remain unmetabolized. The observed antagonistic effect of several sugars on trehalose toxicity suggests that trehalose interferes with some reaction(s) involving sugars.

Table I shows that at the time of blackening of explants fed with trehalose alone (day 4) or with both trehalose and sucrose (day 6), trehalose constituted about 30% of the total soluble sugars in the tissue in both treatments. This suggests that the ratio of

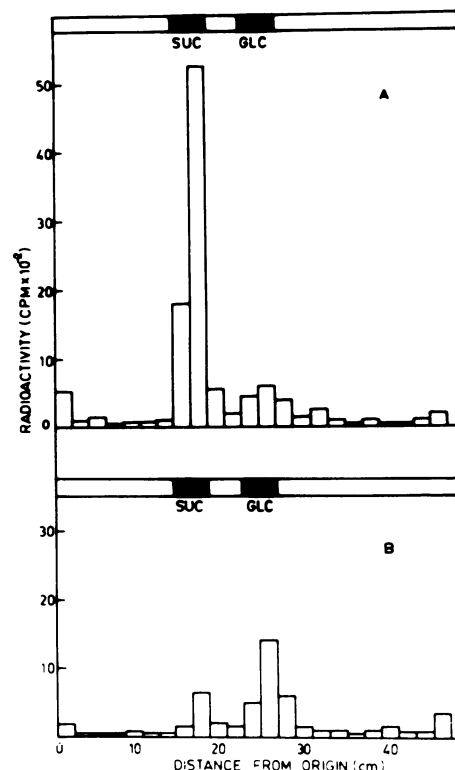


FIG. 5. Chromatography of the neutral fractions from [¹⁴C]glucose-fed shoot tips of *Cuscuta*. The shoot tips were cultured in BM (A) or BM + 2% trehalose (B) for 60 h. After the growth period 3 shoot tip explants (2 cm) from each treatment were fed [¹⁴C]glucose for 2 h. Neutral fraction corresponding to one shoot tip was chromatographed. Positions of sucrose (SUC) and glucose (GLC) in the chromatograms are indicated on top.

concentrations of endogenous trehalose to soluble sugars rather than the absolute concentration of trehalose in the shoot tip may be important in the induction of toxicity.

Although the levels of sugars in the trehalose-fed shoot tips declined, their fate is not clear. Trehalose toxicity was associated with the information of black insoluble substance (13). Presumably, this is a polymerized product of phenolic substances and sugars may have been utilized for its synthesis through the mediation of pentose phosphate pathway and shikimic acid pathway (12).

The fall in the sucrose content of the shoot tip was one of the most pronounced changes upon trehalose feeding. An inhibition of sucrose synthesis has been observed also in mannose-pretreated spinach protoplasts (3). The relationship between the fall in sucrose content and development of blackening in the shoot tip is not known, but a decline in the level of this sugar may become critical. Sucrose plays varied and vital roles in plants (8), among which is its postulated role as a source of UDP-glucose required in the biosynthesis of cellulose (7, 10). As reported earlier (13), trehalose toxicity in *Cuscuta* is confined to the region of the vine which is involved in elongation growth, *i.e.* the terminal 2.5-cm portion. In a trehalose-fed vine, sucrose deficiency may be more critical to the growing cell than to the mature cell.

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