Phytochemistry, 1980, Vol. 19, pp. 205–209. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0201-0205 $02.00/0

SUCROSE METABOLISM DURING THE GROWTH OF *CAMELLIA JAPONICA* POLLEN

NORIO NAKAMURA*, MAMORU SADO* and YUJI ARAI†

*Department of Biology, Yokohama City University, Yokohama 236, Japan;
†Institute of Applied Biochemistry, The University of Tsukuba, Ibaraki-ken 300-31, Japan

(*Revised received* 6 *June* 1979)

Key Word Index—*Camellia japonica*; Theaceae; pollen growth; sucrose; sucrose synthetase; invertase; wall-bound invertase.

Abstract—The free sugar in the mature pollen grains of *Camellia japonica* is almost all sucrose and the sucrose content decreases rapidly during pollen growth. The activity of soluble invertase increases during culturing and a high constant activity was found at the later stages of pollen tube growth. By contrast, the level of sucrose synthetase activity remains constant during pollen growth and that of wall-bound invertase activity is very low. Cycloheximide has little effect on the activity of these enzymes. Exogenous sucrose or glucose was simultaneously incorporated into the pollen grains when they absorbed water and swelled. The free sugar levels in growing pollen depend on the nature of the exogenous sugar. The sugar metabolism in the pollen at the stage of germination differs from that during tube growth, the latter being particularly influenced by exogenous sugar.

INTRODUCTION

During pollen germination and tube growth, sugars are utilized as respiratory substrates and materials for tube wall and starch synthesis [1–3], and the most useful sugar for pollen growth is generally sucrose [4]. Mature pollen grains from diverse plants contain sucrose as the sugar reserve [5]. In order to elucidate the mechanism of pollen growth, therefore, it is important to examine the properties and levels of the enzymes involved in sucrose metabolism. In a previous paper [6], we reported that sucrose synthetase (EC 2.4.1.13) and invertase (EC 3.2.1.26) occur widely in mature pollen grains, the enzymes varying in optimal pH and temperature and in the effect of metal ions on activity.

Furthermore, it was shown that the mature quiescent pollen grains of *Camellia japonica* contain sucrose but not starch as reserve carbohydrate, that starch granules were also lacking in the grain and tube during pollen growth, and that the most effective exogenous sugars for *in vitro* pollen growth are sucrose and glucose [7].

The purpose of this investigation is to clarify roles of sucrose and the enzymes involved in sucrose metabolism in the developing pollen of *Camellia japonica*. The changes in the activities of invertase and sucrose synthetase and those in the levels of sucrose, glucose, UDP-glucose, G6P and F6P in the pollen cultured on various sugars are reported.

RESULTS AND DISCUSSION

In some cases, pollen grains have been cultured in solution [3, 8, 9]. In our preliminary experiments of pollen-suspension culture, both the germination time and the rate of tube growth of camellia pollen were not simultaneous and tube growth stopped within a few hours. Therefore, in the present study, the acetone-treated pollen grains [10], which had tolerable synchrony in germination time and did not aggregate, were cultured on a sugar–agar–plate (SAP) medium. The percentage germination was 90–95% under standard conditions (Table1). The time of germination of pollen cultured on a sugar-free medium was delayed by adding cycloheximide but the final per cent germination was not affected. These results indicate that the mature pollen grains of camellia can germinate whenever they absorb water but that tube growth is inhibited by this drug.

Soluble and cell wall-bound invertase activities were examined in pollen growing on various sugars. As shown in Fig. 1, the levels of soluble invertase markedly increased in all cases during the stages of germination and early tube growth, and the enhanced activities were maintained till the later stages of tube growth. Similar results were obtained with root disks of sweet potato [11] and lentil epicotyl [12]. It has been assumed that there is *de novo* synthesis of invertase because of its susceptibility to cycloheximide inhibition [11,12]. On the other hand, it has been suggested that cycloheximide can disrupt cellular metabolism in ways other than by inhibiting protein synthesis in higher plant systems [13]. In camellia pollen, the first increase of invertase activity during germination was not inhibited by this drug. However, the second increase during the early tube growth tended to be inhibited by this drug. It is assumed that the increase of invertase activity in the growing pollen

Table 1. Effects of various exogenous sugars on pollen germination and tube elongation of *Camellia japonica*

Incubation time (hr)	Cycloheximide added (5×10^{-5} M)	Germination (%)			Tube elongation (mm)		
		Sucrose	Glucose	Sugar-free	Sucrose	Glucose	Sugar-free
1	−	84±5	72±13	26±17	+	+	+
	+	82±9	81±7	45±26	+	+	+
3	−	91±4	89±5	76±14	1.0±0.1	0.6±0.1	0.2±0.1
	+	86±6	74±20	68±26	0.1±0.1	+	+
6	−	93±3	90±3	92±5	2.7±0.3	2.1±0.1	1.6±0.5
	+	90±3	74±28	73±25	0.3±0.2	+	+
14	−	93±4	90±4	94±1	7.6±0.8	5.8±0.4	3.0±0.6
	+	84±6	90±7	88±4	0.2±0.1	+	+

Pollen grains were cultured on sugar (0.3 M)–agar–plate medium at 25°. +: Tube length was below 0.1 mm long.

of camellia is not due to *de novo* synthesis but due to activation of a latent enzyme.

The wall-bound invertase has been reported to occur in sugar beet roots [14–16], *Haemanthus albiflos* pollen [17], *Convolvulus* callus [18, 19] and sweet potato roots [11]. Little activity of wall-bound invertase was detected in the cell walls of camellia pollen (Fig. 1): the optimal pH was 6.1 and its value was the same as that of soluble invertase. However, its activity was less than 1/100 of that of soluble invertase and did not change during pollen growth. Data presented in Table 3 suggest that exogenous sucrose infiltrated into pollen without hydrolytic cleavage by this enzyme, because the sucrose level in the pollen cultured on a sucrose medium increased with increasing duration of culture.

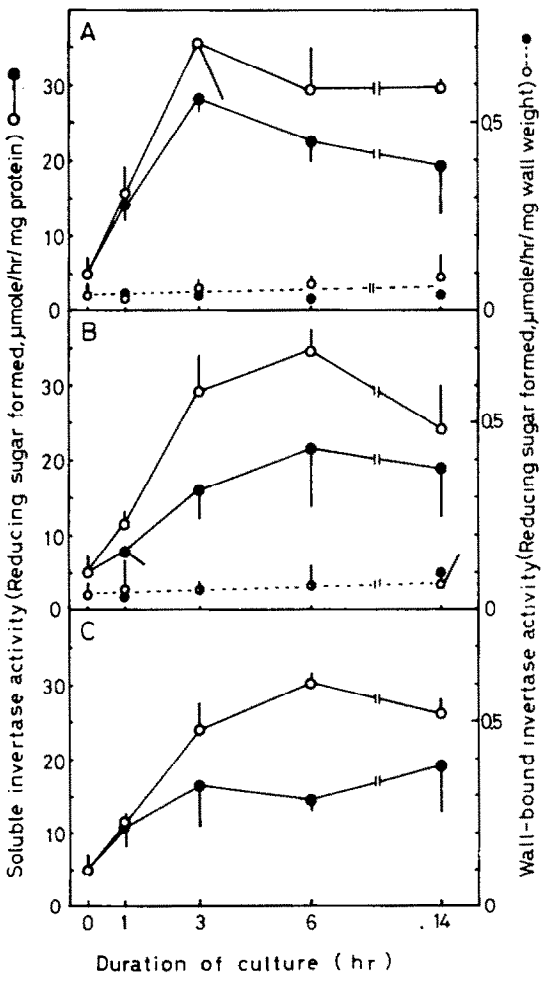

Fig. 1. Changes in the activity of invertase in the growing pollen of *Camellia japonica*. Pollen was incubated on a sucrose (0.3 M) (A), glucose (0.3 M) (B) or sugar-free (C) medium in the presence (●) or absence (○) of 5×10^{-5} M cycloheximide at 25°.

On the other hand, the activity of sucrose synthetase and total protein content before culture were 339± 76 nmol/hr/mg protein and 43±6 mg/10.7×10^{5} pollen grains, respectively. These values did not change during the following 14 hr culture period under each culture condition and also cycloheximide had little effect on the enzyme level and protein content during pollen growth. It has been reported that sucrose synthetase is allosteric for the sucrose cleavage reaction [20, 21] and that the enzyme from potato tuber [22] shows optimal activity for sucrose synthesis at alkaline pH and for sucrose cleavage at acidic pH. In this experiment, the activity of sucrose synthetase was measured in the direction of sucrose synthesis. Therefore, if the activity had been measured in the direction of sucrose cleavage, there might be a higher value. Taking into consideration these facts together with the levels of sucrose, fructose and UDP-glucose in the growing pollen (Tables 2 and 3) and the properties of the enzyme reported previously [6], sucrose synthetase in camellia pollen may in fact take part in the cleavage of sucrose.

Free sugar in the mature quiescent pollen of camellia was mostly sucrose (Tables 2 and 3), and the sucrose level decreased rapidly during germination (Table 3). Subsequently, the sucrose level in the pollen cultured on a sugar-free or glucose medium decreased with the period of tube growth. In the case of culture on sucrose, the pollen grains took up exogenous sucrose when they absorbed water and swelled (Table 3). Consequently, the sucrose level was twice as high as the original sucrose level but it decreased during germination, and then increased again during tube growth (Table 3). However, when cycloheximide was added to the medium, the sucrose level in the growing pollen cultured on the sucrose medium increased slightly and that on the glucose medium did not change until 3 hr of culture. Thus, the sucrose level in the growing

Table 2. Levels of various sugar in the growing pollen of *Camellia japonica*

Medium	Incubation time (hr)	Sugar level (nmol/10.7×10⁵ pollens) G6P	F6P	UDP-glucose	Glucose	Fructose
Sugar-free	0	8±8	4±4	*	250±128	28±22
	2	58±31	12±8	299±76	311±117	133±83
	4	65±46	23±23	449±120	167±111	133±72
	6	27±12	8±8	350±92	106±44	106±89
	8	15±12	*	143±12	111±61	94±72
	14	*	—	120±4	—	—
Glucose	2	158±31	50±8	233±35		
	4	219±96	100±50	399±136		
	6	146±85	62±31	387±88		
	8	88±8	35±12	261±55		
	14	142±100	50±46	125±16		
Sucrose	2	215±115	108±73	307±18		
	4	242±12	88±23	451±51		
	6	269±31	138±31	355±76		
	14	69±12	12±12	143±30		
Fructose	4	58±12	12±4	—	2444±378	
	6	19±4	*	—	3661±539	

Pollen grains (0.1 g) were incubated and all growing pollen was collected. Sugar content was determined according to the enzymatic method [31, 32].

*Undetectable.

pollen depends on the kind of sugar supplied exogenously. During pollen growth, sucrose level decreased with duration of culture, whereas invertase activity increased. Similar results were obtained with the disks of sugar beet roots [23], sweet potato [24] and carrot roots [25]. Sucrose was presumably cleaved by invertase because of the increase in glucose and fructose, and the glucose and fructose formed were utilized as an energy source and as material of pollen tube walls. The sucrose level in the pollen cultured on a sugar-free medium was almost completely consumed within 6–14 hr, this period corresponding to the time when pollen growth stopped. From this result, it may be concluded that the pollen growth stopped due to a shortage of nutrients.

The levels of reducing sugars in the pollen cultured on sugar media increased during pollen tube growth and were affected by cycloheximide. In the case of

Table 3. Sugar levels in the growing pollen of *Camellia japonica*

Medium	Incubation time (hr)	Sugar level (μmol/10.7×10⁵ pollens) Sucrose		Reducing sugar	
Sugar-free	0	47.7±2.9		0.6±0.6	
	1	8.2±15	(9.1±2.0)	1.7±0.6	(1.7±0.6)
	3	4.4±0.9	(7.9±1.5)	3.3±0.6	(1.1±0.6)
	6	2.3±0.3	(5.3±1.5)	4.4±1.1	(1.1±0.0)
	14	1.5±0.0	(4.3±0.6)	3.9±1.1	(1.1±0.0)
Sucrose	0.05	90.9±5.3		2.2±0.6	
	1	70.5±1.5	(94.4±7.9)	3.9±0.6	(2.8±0.6)
	3	71.1±13.2	(75.4±8.8)	46.1±12.2	(4.4±0.6)
	6	93.6±10.5	(81.6±12.6)	80.0±14.4	(6.7±1.1)
	14	116.7±9.1	(88.3±10.8)	88.3±9.4	(37.8±9.4)
Glucose	0.05	49.1±1.2		56.7±4.4	
	1	41.2±1.5	(52.0±4.7)	114.4±12.2	(97.2±5.6)
	3	29.5±1.5	(54.1±6.4)	136.7±3.3	(98.9±14.4)
	6	17.3±2.6	(40.6±6.7)	142.2±31.7	(129.4±5.6)
	14	7.9±0.6	(15.8±1.2)	117.8±16.7	(71.7±6.7)

Pollen grains (0.1 g) were cultured on sugar (0.3 M) media in the presence (the data are shown in parentheses) or absence of 5×10^{-5} M cycloheximide at 25°.

culture on a sugar-free medium, however, they were very low (Tables 2 and 3).

The levels of G6P and F6P increased during germination and early tube growth, and the G6P level was 2–5 times as high as the F6P level throughout (Table 2). The levels of G6P and F6P in the pollen cultured on sucrose or glucose were higher than those in the pollen cultured on fructose or in the absence of sugar. These differences between sugar phosphate levels may relate to the levels present during pollen growth. On the other hand, UDP-glucose could not be detected in the quiescent pollen grain (Table 2). The UDP-glucose level, however, increased rapidly during germination and early pollen tube growth. A change in UDP-glucose level during the germination of the pollen cultured on a sugar-free medium was similar to that of the pollen cultured on the different sugars. This suggests that UDP-glucose is formed from endogenous sucrose. Since UDP-glucose is an essential precursor of pollen tube wall polysaccharide, the UDP-glucose level in the growing pollen may influence tube growth. However, G1P, the substrate of UDP-glucose, was not detectable in the pollen cultured on these sugars and in the pollen at any growth stage, probably due to its rapid metabolism.

The dry weight of growing pollen on sugar media changed little during the first 3 hr of culture, and then it increased with pollen tube growth; and yet that of pollen cultured on a sugar-free medium decreased during pollen growth although the pollen tube grew (Table 4). Cycloheximide inhibited this increase in dry weight. After 6 hr, the tube lengths of pollen cultured under various conditions were about equal but the dry weight of pollen differed greatly from one to another. The dry weight was approximately the total sum of the weights of free sugars, protein and pollen walls. Since the protein content is nearly constant during pollen growth, the changes in dry weight are due to changes in free sugar levels and to increases in tube wall weights.

Dickinson [8, 26] has found that the respiration rate in germinating lily pollen varies according to whether there is rapid starch accumulation, tube initiation or tube elongation. The present data show that the levels of sugars and invertase activity in the growing pollen of camellia change significantly before and after early tube growth. It thus appears that sugar metabolism alters during pollen growth and that during tube growth is particularly influenced by exogenous sugar. Endogenous sucrose and then exogenous sucrose or glucose are utilized for pollen germination and tube growth, because most of the sugar in the mature pollen grains is sucrose. The decrease in the sucrose level is correlated with increase in the activity of soluble invertase. By contrast, the activity of sucrose synthetase does not increase, and is much lower than that of invertase. Hydrolysis of sucrose is thus catalysed by invertase. Nevertheless, it is also possible that sucrose synthetase may be related to the hydrolysis of sucrose and concomitant synthesis of UDP-glucose. Glucose and fructose formed from sucrose are converted to other sugars. Tupý [27] has indicated that the stimulatory effect of sucrose on tube growth of apple pollen may be attributed to the β-D-fructofuranose moiety of sucrose. In camellia pollen, however, exogenous fructose is not effective for tube growth [7] and also the levels of G6P and F6P in pollen growing on a fructose medium are very low (Table 2). Besides, the data presented here suggest that glucose is utilized predominantly for tube growth, although a high glucose level in the growing pollen cell is not effective for tube growth (Tables 1 and 3).

EXPERIMENTAL

Materials and culture condition. Anthers taken from ripe flowers of *Camellia japonica* were dried for 24 hr in an oven at 30° and the pollen grains therein were sieved and stored over Si gel at −15° until use. The pollen grains were cultured on a SAP medium at 25° after washing with Me_2CO to remove lipids adhering to their surface; this treatment has little effect on pollen germination and tube growth [10].

Preparation of enzymes. Pollen grains (0.5 g) suspended in K-Pi buffer (5 mM, pH 7.0) were disrupted in a French press

Table 4. Changes in dry weight of the growing pollen of *Camellia japonica*

Incubation time (hr)	Cycloheximide added (5×10^{-5} M)	Dry weight (mg/10.7×10^5 pollens) Sucrose medium	Glucose medium	Sugar-free medium
0.05		95.0±4.1	88.0±2.7	91.0±2.0*
1	−	97.5±4.0	85.4±0.3	74.4±4.0
	+	93.1±6.9	91.4±1.8	74.3±5.2
3	−	98.4±11.4	79.7±0.2	70.7±4.0
	+	99.9±13.6	89.6±1.7	70.7±0.9
6	−	137.3±8.9	101.4±13.7	72.0±2.9
	+	106.6±12.3	81.4±1.7	63.0±2.2
14	−	163.6±7.6	142.0±6.7	63.7±1.7
	+	101.0±7.9	77.7±2.8	56.0±2.8

Pollen grains (0.1 g) were cultured at 25°. After the growing pollen was collected and the medium removed through filter paper, it was dried in an oven at 130° for 3 hr.

*Unincubated pollen grains.

(1300–1500 kg/cm^2) and the growing pollen collected and homogenized in a Potter homogenizer. The homogenate was centrifuged, the supernatant was collected and the cell debris was extracted twice by the above procedure. The combined supernatant was adjusted to a 100 ml vol. with the same buffer and $(NH_4)_2SO_4$ was added to 50% saturation. The ppt. was collected by centrifugation (10 000 g, 30 min) and dissolved in 5 ml of the same buffer. The soln was dialyzed against the same buffer and used as a crude soluble invertase preparation. The recovery of invertase activity was 78% that of initial supernatant. The cell debris collected was subjected to the same extraction procedure until soluble invertase activity could not be detected in the supernatant. The ppt. obtained was used for the preparation of wall-bound invertase. Sucrose synthetase was extracted as described for soluble invertase, except Tris buffer was used instead of K-Pi buffer. The recovery of the enzyme was 85%. The crude enzyme soln obtained showed invertase activity. However, the invertase activity was lowered in the presence of Tris buffer and $MnCl_2$, and almost completely inhibited at the alkaline pH of the assay for the synthetase activity. Therefore, the crude enzyme soln was used in this expt.

Enzyme assay. Invertase activity was determined by reaction with 3,5-dinitrosalicylic acid [28]. After the reaction mixture containing 10 μmol sucrose, 80 μmol K-Pi buffer (pH 6.1) and 0.3 ml enzyme soln in a total vol. of 1 ml was incubated at 30°, the reducing sugar formed was measured colorimetrically. In the case of wall-bound invertase, 1 ml of pollen wall suspension was used as the enzyme in a total vol. of 3 ml. After being incubated at 30°, the reaction mixture was centrifuged (2500 g, 10 min) and the reducing sugar in the supernatant was measured. In the assay of sucrose synthetase activity, the reaction mixture containing 0.5 μmol UDP-glucose, 2 μmol fructose, 1 μmol $MnCl_2$, 10 μmol Tris buffer (pH 8.5) and 50 μl of an enzyme soln in a total vol. of 200 μl was incubated at 37°. The reaction was stopped by heating at 100° for 5 min and sucrose formed was determined by the method of ref. [29]. Protein content was measured by the method of ref. [30].

Extraction of sugar. Pollen was suspended in 10 ml 80% EtOH and immediately heated for 5 min at 100°. After cooling, the suspension was homogenized with a Potter homogenizer and the homogenate was centrifuged (10 000 g, 10 min, 20°). The ppt. was repeatedly extracted as described above, until the anthrone reaction of the supernatant became negative. All the supernatants were combined and evapd to dryness. The residue was dissolved with H_2O and the soln was used for sugar analysis.

Measurement of sugar content. Sucrose and fructose were determined according to the method of ref. [29]. UDP-glucose, G6P, F6P, G1P, glucose and fructose were determined according to the enzymatic analysis of Bergmeyer *et al.* [31, 32]. In the measurement of UDP-glucose, the reaction mixture contained 100 mM glycine–NaOH buffer (pH 8.7), 0.5 mM NAD, 25 μg UDP-glucose dehydrogenase and 0.5 ml of sample in a total vol. of 2 ml. With other sugars, the reaction mixture contained 100 mM Tris buffer (pH 7.5), 0.5 mM NADP, 5mM $MgCl_2$, 2.5 mM ATP, 5 μg G6P dehydrogenase, 15 μg hexokinase, 10 μg phosphoglucoisomerase, 10 μg phosphoglucomutase and 0.5 ml of sample soln in a total vol. of 2 ml. The reaction was carried out at 25° and the increase of NADH or NADPH was determined with a double beam spectrophotometer.

REFERENCES

1. O'Kelly, J. C. (1955) *Am. J. Botany* **42**, 322.
2. Iwanami, Y. (1959) *J. Yokohama City Univ.* **16**, 1.
3. Nygaard, P. (1977) *Physiol. Plant.* **37**, 206.
4. Linskens, H. L. and Kroh, M. (1970) in *Current Topics in Developmental Biology* (Moscona, A. A. and Morroy, A., eds.) p. 89. Academic Press, New York.
5. Hrabětová. E. and Tupý, J. (1964) in *Pollen Physiology and Fertilization* (Linskens, H. L., ed.) p. 95. North-Holland, Amsterdam.
6. Nakamura, N., Arai, Y. and Iwanami, Y. (1975) *Nippon Nogei Kagaku Kaishi* **49**, 469.
7. Nakamura, N. (1978) *J. Yokohama City Univ. Biol. Ser.* **5**, 1.
8. Dickinson, D. B. (1967) *Physiol. Plant.* **20**, 118.
9. Singh, M. B., Malik, C. P. and Thapar, H. (1978) *Plant Cell Physiol.* **19**, 678.
10. Iwanami, Y. and Nakamura, N. (1973) *Stain Technol.* **47**, 137.
11. Murata, T. (1973) *Nippon Nogei Kagaku Kaishi* **47**, 557.
12. Seitz, K. and Lang, A. (1968) *Plant Physiol.* **43**, 1075.
13. MacDonald, I. R. and Ellis, R. J. (1969) *Nature* **222**, 791.
14. Masuda, H. and Sugawara, S. (1973) *Nippon Nogei Kagaku Kaishi* **47**, 147.
15. Vaughan, D. and MacDonald, I. R. (1967) *J. Exp. Botany* **18**, 578.
16. MacDonald, I. R., Bacon, J. S. D., Vaugh, D. and Ellis, R. J. L. (1966) *J. Exp. Botany* **17**, 822.
17. Lendzian, K. and Schafer, E. (1972) *Phytochemistry* **12**, 1227.
18. Klis, F. M. and Hak, A. (1972) *Physiol. Plant.* **26**, 364.
19. Klis, F. M., Dalhuizen, R. and Sol, K. (1974) *Phytochemistry* **13**, 55.
20. Murata, T. (1971) *Agric. Biol. Chem.* **35**, 1441.
21. Murata, T (1972) *Agric. Biol. Chem.* **36**, 1815.
22. Pressey, R. (1969) *Plant Physiol.* **44**, 759.
23. Bacon, J. S. D., MacDonald, I. R. and Knight, A. H. (1965) *Biochem. J.* **94**, 175.
24. Matsushita, K. and Uritani, I. (1974) *Plant Physiol.* **54**, 60.
25. Ricard, C. P. P. and ap Rees, T. (1970) *Phytochemistry* **9**, 239.
26. Dickinson, D. B. (1968) *Plant Physiol.* **43**, 1.
27. Tupý, J. (1962) *Biol. Plant.* **4**, 69.
28. Borel, E., Hostettler, F. and Denel, H. (1952) *Helv. Chim. Acta.* **35**, 115.
29. Rorem, E. S., Walker, H. G. and McCreadl, R. M. (1960) *Plant Physiol.* **35**, 269.
30. Gornall, A. G., Bardwill, C. S. and David, M. M. (1949) *J. Biol. Chem.* **177**, 751.
31. Bergmeyer, H. U. and Michal, G. (1974) in *Methods of Enzymatic Analysis* Bergmeyer, H. U., ed.) Vol. 3, p. 1233. Academic Press, New York.
32. Seifert, L. (1966) in *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 8, p. 248. Academic Press, New York.