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Analyses of the Carbohydrates Leached During Drying and Wetting of Sclerotia of *Sclerotinia sclerotiorum* (Lib) De Bary

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ABSTRACT. Sclerotia of *Sclerotinia sclerotiorum* (Lib) De Bary were exposed to different relative humidities ranging from zero to 100% for different periods of time up to 168 hr. Leaching of carbohydrates occurred after sclerotia were wetted. At zero and 33% relative humidities leaching of carbohydrates was highest and increased with period of exposure. Quantitative estimation of carhohydrates released showed that leaching was in the order trehalose, mannitol, glucose with fructose in trace amounts. On drying and rehydration of sclerotia at two relative humidities (zero and 100%) severe carbohydrate leaching occurred. Quantities leached were found to increase with the period of incubation at zero relative humidity but decreased with the period of incubation at 100% relative humidity.

It is well-known that most sclerotium-forming fungi, which cause important plant diseases, can survive in the soil for long periods even under adverse environmental conditions (Coley-Smith and Cooke 1971, Willetts 1971). While attempts to control soil-borne pathogens by biological means have been largely unsuccessful (Baker 1968). Smith (1972 b, c) showed that sclerotia of *Sclerotium rolfsii* Sacc. and *Sclerotium cepivorum* Berk. could be eliminated from soil. They were first dried for a short period and then remoistened. This elimination was attributed to the leakage of nutrients from the sclerotia which were then colonized by other microorganisms in the soil. Furthermore, it was suggested that this treatment had important implications in the biological control of other plant diseases caused by sclerotium-producing fungi. Similar results have been obtained by Javed and Coley-Smith (1973) and by Coley-Smith *et al.* (1974) with *Sclerotium delphinii* Welch (a form of *Sclerotium rolfsii*) and *Morcophomina phasedina* but not with *S. cepivorum*, *Botrytis cinerea* and *Botrytis tulpiae* (Papavizas 1977).

It has been shown that microbial activity increases when dry sclerotia of *Sclerotium cepivorum* are placed in sterile soil (Dickinson and Coley-Smith 1970, Coley-Smith and Dickinson 1971, Smith 1972c, Papavizas 1977). Similar results have also been obtained with *Sclerotium rolfsii* (Smith 1972b), *Sclerotinia sclerotiorum* and *S. minor* (Smith 1972c), and *Macrophomina phaseolina* (Papavizas 1977).

Drying of sclerotia of *Sclerotium rolfsii, Sclerotinia sclerotiorum* and *S. minor* stimulates their germination in moist soil in the absence of the host plants or a suitable substrate but this does not occur with *S. cepivorun* (Smith 1972a, c). Coley-Smith (1959) found that a large proportion of air-dry *Sclerotium cepivorum* sclerotia survived for 4 years in field soil.

It is clear that wetting of dried sclerotia is accompanied by leakage of substances (Dickinson and Coley-Smith 1970, Coley-Smith and Dickinson 1971, Smith 1972b, c, Coley-Smith *et al.* 1974, Javed 1977b, Papavizas 1977). Although much attention has been paid to the physiological factors affecting formation and composition of sclerotia, little has been published on the nature of substances leached during imbibition (Coley-Smith and Dickinson 1971, Smith 1972b, c, Coley-Smith *et al.* 1974, Javed 1977b). Although some observations have been made on the exudates of sclerotia these refer only to the early stages of their development (Cooke 1969). Thus, it seems that no quantitative analysis has been made of sugars leached from sclerotia except for that of Smith (1972b).

Since the leakage of nutrients from sclerotia probably plays an important role in biological control of sclerotia, the aim of this study was first to investigate the effect of drying and wetting on the loss of water from sclerotia of *Sclerotinia sclerotiorum* and, in addition, to investigate the effect of redrying on exudation. A further aim was to identify and measure the major carbohydrates leached.

Material and Methods

1. Preperation of Sclerotia and Leachates

The isolate used was obtained from Prof. D. Le Tourneau, Department of Bacteriology and Biochemistry, University of Idaho, USA. Sclerotia of *Sclerotinia sclerotiorum* were grown on PDA at 25°C in an incubator and 25-45 days old sclerotia were then used for experiments. Cultures were maintained on Difco potato-dextrose agar (PDA) at 20-24°C and transferred at regular intervals. Samples of sclerotia were placed in desiccators containing either 1*l* of a saturated solution of $MgCl_2 \cdot 6H_2O$ or of a saturated solution of NaCl to provide 33 or 76% RH at 20°C (Winston and Bates 1960). Active desiccant silica gel (self indicating) and water were used to provide 0 and 100% RH, respectively. Sclerotia (for age see each experiment) were placed in uncovered 50 mm plastic containers (small petri dishes) and were exposed to the four RH values for the time indicated in

each experiment. At various intervals, 1000-2000 mg of sclerotia were removed from each desiccator and the percent weight loss or gain was determined together with percentage water loss by drying the sclerotia in an oven at 90°C for 24 hr and re-weighing (Trevethick and Cooke 1973). A sub-sample of sclerotia was left at 90°C for 24 hr in order to determine the dry weight (control).

In experiments on rehydration, 45-day old sclerotia were stored at 0 RH and transferred to the incubator at 24°C for one week before the experiment. After this time, sclerotia were exposed to 0 and 100% RH. Samples were then examined for water loss, weight loss and leakage.

Leaching assays were carried out at various intervals on 1000-2000 mg of sclerotia removed from the four RH regimes and placed in 50 ml of sterile distilled water for 24 hr at either 20 or 15°C. To determine the amount of leached substances the samples of 20 ml of the above solution (containing leachate) were transferred to aluminium containers for oven-drying at 90°C for 24 hr and re-weighed. A further 25 ml of the leachate was used for either qualitative determination of the component sugars or quantitative analysis of the major carbohydrate leached. The pH of the leachate samples was determined at 24 hr or at zero time (as a control).

2. Sugar Determinations

Paper Chromatography

Leachate was concentrated *in vacuo* in a rotary evaporator at less than 40°C and made to 10-25 ml with 20% ethanol (Wang and Le Tourneau 1971). Carbohydrates were tentatively identified by one-dimensional descending paper chromatography using Whatman paper No. 1. The solvent systems employed were *n*-butanol-pyridine-water (6:4:3) and methyl ethyl ketone-acetic acid-water saturated with boric acid (9:1:1) (Le Tourneau 1966). The carbohydrate spots were developed using the silver nitrate-sodium ethoxide method (Trevelyan *et al.* 1950), aniline hydrogen-phthalate (Le Tourneau 1966), and resorcinol-HCl (Yem and Willis 1954).

Gas-Liquid Chromatography of TMS Derivatives

Quantitative analyses were made using GLC of TMS derivatives (Sweeley *et al.* 1963). Qualitative identification was also employed to confirm the results which were obtained with paper chromatography. The leachate was deionized by adding a small quantity of a mixture of Amberlite ion-exchange resins IR-120 (H^+) and IR-45 $(OH)^-$, followed by filtration.

The leachate was finally reduced to dryness *in vacuo* in a rotary evaporator at less than 40°C, taken up in 2 ml of 80% ethanol and stored (Cooke 1969). The apparatus used was a Pye Series 104 Dual Column Analytical Gas Chromatograph,

Table 1. Effects of exposure of sclerotia (2000 mg) of *Sclerotinia sclerotiorum* to zero, 33%, 76%, 100% RH and room conditions on % water loss and leaching of substances.

Exposure time (hr)	RH	Wt. after exposure (mg)	Dry wt. (mg)	loss	% Water loss	% moisture contents	Wt. before leaching (mg)	pH	% Water loss	Dry wt. (mg)	Substances leached
24	Zero	1245	920	37.3	69.9	26.1	1015	3.9	49.3	840	Glucose*
	33%	1195	939	53.1	75.9	21.4	1216	4.5	39.2	907	Glucose*
	76%	1673	940	16.4	30.8	43.8	1682	4.4	15.9	885	Glucose*
	100%	1965	911	1.8	3.2	53.6	2024	4.2	1.2	1084	Glucose*
72	Zero	1142	1070	42.9	92.3	6.3	954	6.1	52.3	830	Glucose* Mannitol Trehalose
	33%	1020	921	49.0	90.8	3.7	1046	4.2	47.7	937	Glucose* Fructose"
	76%	1150	900	42.5	77.3	21.7	1184	4.4	40.8	920	Glucose* Fructose"
	100%	2052	1075	2.6	5.6	47.6	1910	4.9	4.5	892	Glucose"
120	Zero	1070	1016	46.5	94.5	5.0	952	5.5	52.4	821	Glucose*
	33%	987	897	50.7	91.8	3.1	1018	3.7	49.1	918	Fructose Glucose* Mannitol
	76%	1095	895	45.3	81.9	18.3	1135	3.6	43.3	926	Glucose
	100%	1992	1010	0.4	0.8	49.3	1852	4.1	7.4	892	Glucose"

168	Zero	1068	1020	46.6	95.1	4.5	1075	6.6	46.3	925	Glucose* Trehalose Fructose Mannitol
	33%	1014	928	49.3	92.0	8.5	990	4.5	50.5	892	Glucose* Mannitol
	76%	1106	912	44.6	82.2	17.5	1116	4.4	44.2	910	Glucose
	100%	2082	1057	4.1	8.7	49.2	2059	5.1	3.0	1040	–
24	At Room Conditions	1000	910	50.0	91.7	9.0	1000	5.1	50.0	880	Glucose* Trehalose Mannitol
72		989	910	50.6	92.8	8.0	995	5.3	50.3	877	Glucose* Trehalose Mannitol Fructose
120		982	919	54.1	94.2	6.4	962	5.3	51.9	831	Glucose Trehalose Mannitol Fructose
168		1022	955	48.9	93.6	10.1	1025	5.8	48.8	914	Glucose* Trehalose Mannitol Fructose

* Amounts increasing with the lower RH

" Trace amounts. Original fresh weight taken was 2 g.

with flame ionization detectors and glass columns, in conjunction with a Leeds and Northrup 'Speedomax W' Recorder. The 152 cm columns were packed with 3% S.E. 30 on chromosorb 100-120 mesh. All analyses were carried out using the methods of Holligan and Drew (1971).

Results

1. The Effect of Exposure of Sclerotia to Controlled RH on Water Loss and Final Moisture Content

In one experiment, sclerotia (age 19 days) were exposed to zero, 33%, 76% and 100% RH for 24, 72, 120 and 168 hr, respectively. One sample was kept at room conditions (air dried) for the same period. The percentage water loss, pH value as well as substances leached were determined. Results also enlisted in Table 1. From Table 1, it can be seen that in all treatments, both weight loss and water loss decreased with increasing RH's. The moisture content, however, increased with increasing RH in all cases. The substances leached were trehalose, mannitol, glucose (which was present in trace amounts which increased with time) and fructose, which was present in trace amounts.

2. Effect of Drying Sclerotia at Zero RH and Rewetting on Weight Loss and Leaching of Carbohydrates

In this experiment, sclerotia (age 24 days) were exposed to 100% RH for 20 hr and then stored in a desiccator of zero RH for 5 days. Then, sclerotia were placed in water, and at intervals of time (see Table 2) were weighed, and after 24 hr at 90°C their dry weight after leaching was determined. The carbohydrates leached were also determined by paper chromatography. Results are listed in Table 2. It is clear that mainly trehalose, mannitol and glucose were leached from sclerotia.

3. Effects of Rehydration of Sclerotia on Substances Leached and Percentage Water Loss

Here, 45-day old sclerotia were used, dried for one week at zero RH and incubated at two relative humidities (Zero and 100%) for 24, 72, 120 and 168 hr. Figure 1a shows the effect of rehydration on the amount of substances leached with time. It is very clear from the figure that substances leached increased with time at zero RH while at 100% RH leached substances decreased with incubation time. Water loss, however, followed the same pattern in that it increased with incubation time at zero RH but there was water gain at 100% RH (Fig. 1b).

Table 4 contains results of the above experiment from which Fig. 1a and b were drawn.

Table 2. Weight loss and carbohydrates leached from sclerotia of *Sclerotinia sclerotiorum* (2000 mg) previously dried at zero relative humidity.

Period of leaching (hr)	Wt. after exposure (mg)	Dry wt. after leaching (mg)	% weight loss	Wt. of substances leached (mg)	pH Value	Carbohydrates in leached water
1	960	905	54.8	4.5	5.5	Glucose, Trehalose
2	963	904	54.8	7.5	5.6	Glucose, Trehalose, Mannitol, Fructose
4	960	892	55.4	15.5	5.6	Glucose, Trehalose, Mannitol
6	965	885	55.8	20.8	5.7	Glucose, Trehalose, Mannitol
8	960	876	56.2	25.5	5.6	Glucose, Trehalose, Mannitol
12	960	864	56.8	33.5	5.6	Glucose, Trehalose, Mannitol
16	961	872	56.4	35.3	5.6	Glucose, Trehalose, Mannitol
18	961	860	57.0	36.8	5.7	Glucose, Trehalose, Mannitol
20	950	874	56.3	42.3	5.6	Glucose, Trehalose, Mannitol
24	963	873	36.4	34.5	5.5	Glucose, Trehalose, Mannitol

Original fresh weight was 2 g.

Table 3. The effect of exposure of sclerotia of *Sclerotinia sclerotiorum* to zero RH on the total leachate quantitative carbohydrate estimations after various periods of exposure.

Time of exposure (hr)	Total amount leached (mg)	Soluble carbohydrates leached (mg)			Total Soluble Carbohydrates (mg)	Soluble carbohydrates leached (mg/g dry wt.)			% Total Soluble carbohydrates mg/g wt.	% Non-sugars	Non-sugars mg/g dry wt.	Total leachate mg/g dry wt.	Dry wt. of sclerotia after leaching (mg)
		Trehalose	Mannitol	Glucose		Trehalose	Mannitol	Glucose					
24	15.8	2.5	2.0	1.3	5.8	2.8	3.2	1.4	6.4	1.1	11.5	17.6	899.0
48	73.4	7.8	4.5	3.5	15.8	9.1	5.2	3.5	17.8	6.7	67.0	85.3	861.1
72	77.8	16.0	9.1	5.9	31.0	18.3	10.4	6.7	35.4	5.3	53.4	88.9	875.5
96	72.5	27.0	13.8	10.3	51.1	30.8	15.7	11.7	46.5	2.5	25.1	84.6	857.8

Table 4. Effects of exposure of sclerotia of *Sclerotinia sclerotiorum* to two relative humidities (zero and 100% RH) to 24, 48, 72 and 76 hr, respectively.

RH	Time of exposure (hr)	Wt. after exposure (mg)	% Water loss or gain (mg)	Dry wt. (mg)	% Moisture content	Wt. after leaching (mg)	Water loss or gain (mg)	pH	Wt. of amount leached (mg)	Dry wt. after leaching (mg)
Zero RH	24	546.7	46.5	501.4	8.3	1024.0	90.9	5.3	15.8	899.0
	48	516.0	49.0	483.0	6.4	996.0	93.6	6.0	73.4	861.1
	72	512.0	49.4	484.2	5.6	990.2	94.4	6.3	77.8	875.5
	96	502.8	50.1	482.7	4.0	977.6	96.1	6.3	72.5	857.8
100% RH	24	929.4	6.3	476.8	48.7	1906.5	13.5	5.0	–	925.4
	48	953.0	4.6	483.5	49.3	1915.0	9.1	5.4	1.9	926.0
	72	865.2	12.8	489.7	43.4	1769	26.4	4.9	1.4	937.6
	96	872.1	13.2	494.6	43.0	1737.0	26.0	5.0	–	933.8

Original fresh weight (sclerotia) was 1 g.

Original fresh weight (sclerotia) was 2g for GLC assay.

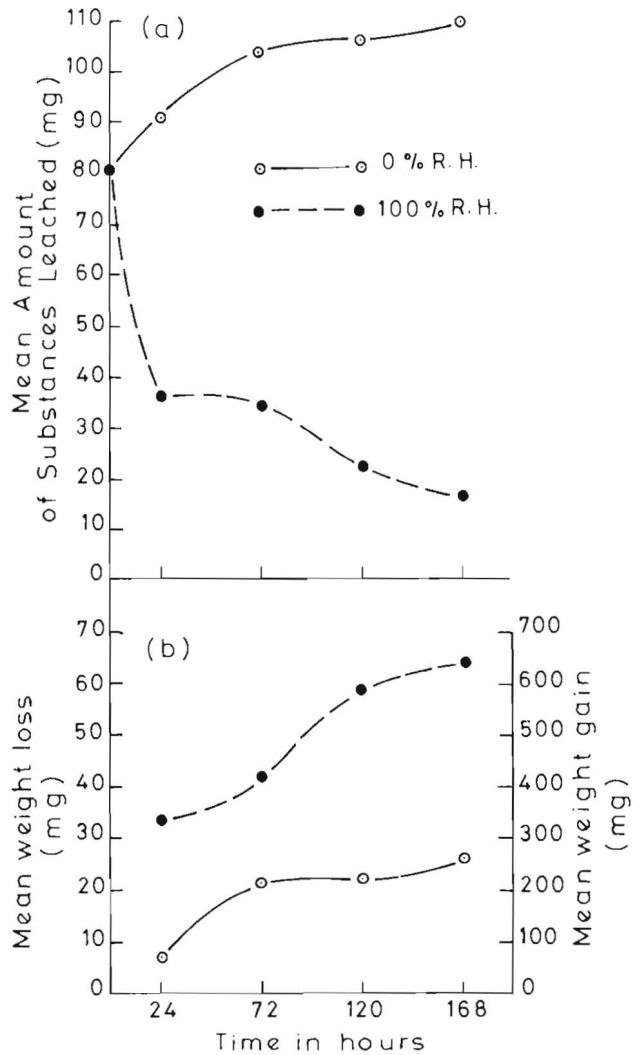

Fig. 1a. The effect of rehydration of sclerotia of *Sclerotinia sclerotiorum* on the amount of substances leached with time.

b. The effect of rehydration of sclerotia on the mean weight loss as a function of time.

4. Amounts of Major Carbohydrates Exuded on Exposure of Sclerotia to Two RH Values

Here, 35-day old sclerotia were grown on PDA. Then samples of 1 g were taken for determination of dry weights and 2 g used for exposure of sclerotia at two RH values (zero and 100%). At 24, 48, 72 and 96 hr, samples of leachate were

taken from the zero % RH samples and carbohydrates present determined quantitatively using GLC. Results are given in Tables 3 and 4. From Table 3 it is evident that the total amount of substances leached from sclerotia generally increased with incubation time. Individual sugars as well as total carbohydrates increased progressively with incubation time.

Discussion

Some workers have reported the survival of sclerotia after periods of drying, while others have reported the opposite. Willetts (1971) found that sclerotia from different sources and of different types survived under adverse environmental conditions for long periods. Javed (1977a) using five sclerotium-forming fungi, found that over a period of three years, there was no significant drop in numbers of *Rhizoctonia tuliparum*, *Sclerotinia gladioli* and *Sclerotium cepivorum*. Sclerotia of *Sclerotium delphinii* and *Botrytis tulipae* failed to survive for the full period. Papavizas (1977) showed that exposure of sclerotia of *Macrophomina phaseolina* and of *Sclerotium cepivorum* for up to 55% RH for several weeks did not reduce their germinability on agar. However, exposure to 78% RH caused high loss of germinability in *M. phaseolina* and complete loss in *S. cepivorum*. Trevethick and Cooke (1973) in desiccation experiments on sclerotia of *Sclerotinia sclerotiorum*, *Sclerotium delphinii* and *S. rolfsii* demonstrated that the sclerotium rind did not effectively restrict water loss. Their observations on rates of rehydration showed that drying of the outer sclerotial tissues did not make them impermeable to water. Cooke (1971) reported that loss of weight in *S. sclerotiorum* at zero RH suggested that the rind formed during growth and maturation was freely permeable to water.

However, Smith (1972a) found that drying sclerotia of *S. rolfsii* for short periods limited their survival in remoistened soil. The dried sclerotia were colonized by other micro-organisms and rotted within two weeks in remoistened soil.

Many workers have reported the chemical composition of sclerotia of various fungi (Le Tourneau 1966, Coley-Smith and Dickinson 1971). Leakage of carbohydrates has been reported for sclerotia of some fungi (Cooke 1969, Smith 1972b) and in some higher plant seeds (Simon and Raja Harun 1972). The mechanism for leakage may be the same in both of them. The nature of substances leached from sclerotia of *S. sclerotiorum* reported in this study are similar to those substances reported in the composition of sclerotia of the same fungus.

In the present study, the quantity of leached substances was higher at first and then declined with incubation time when dried sclerotia were rewetted for various periods. The carbohydrates determinable by GLC, namely trehalose, mannitol and glucose, were found to increase quantitatively with time of incubation of dried sclerotia at zero RH.

Other workers have made qualitative studies on carbohydrates and other com-

pounds composing and leaking from sclerotia. Le Tourneau (1966) found that trehalose and mannitol constituted 6-7% of the dry weight of sclerotia of *Sclerotinia sclerotiorum* grown on glucose-mineral salts agar. Cooke (1969), also using sclerotia of *S. sclerotiorum* during their early development, found that they mainly secreted trehalose, mannitol, glucose and traces of a compound identified as inositol. Mannitol content decreased with development. Cooke and Mitchel (1969) found trehalose and mannitol to be the major soluble carbohydrates of sclerotia of *Claviceps purpurea*, *C. nigricans* and *Sclerotinia curreyana*. Huang (1983) showed leakage of amino acids which were greater in *Sclerotinia sclerotiorum* abnormal sclerotia than normal ones.

Hydrocarbons, free fatty acids and amino acids have been qualitatively and quantitatively identified in sclerotia of *Sclerotinia sclerotium* (Weete *et al.* 1970, Huang 1983). Wang and Le Tourneau (1977) grew *S. sclerotium* in media of various carbon sources. They reported trehalose, mannitol and small quantities of glucose or fructose in sclerotia from all carbon sources. Galactitol or pentitols occurred in sclerotia when the fungus was grown on galactose and oligosaccharides containing galactose or corresponding pentose sugars. Acid hydrolysates contained glucose, galactose, mannose and traces of ribose and mannose. Exudates of *S. sclerotiorum* contained carbohydrates, proteins, lipids, free ammonia, salts, organic acids and enzymes (Colotelo 1973, Colotelo *et al.* 1971, Cooke 1969, Jones 1970). Also Christias (1980) found that *Sclerotium rolfsii* contained more proteins than carbohydrates.

The components of leachate in this study of *S. sclerotiorum* were carbohydrates (trehalose, mannitol and glucose) and non-sugar substances (organic and amino acids, pigments and other materials). Amounts of soluble carbohydrates were 6.35% (dry weight) when sclerotia were exposed to 96 hr at zero RH 6. At the same time, the percentage of non-sugars substances was 2.5% (dry weight). The amounts leaking from fungal sclerotia are sufficient to provide an energy source for the growth of other microorganisms. If this occurred in the soil, then leakage from sclerotia into the soil would stimulate their microbial breakdown.

This study has, therefore, reported for the first time details of the quality and quantity of alcohol-soluble carbohydrates leached from dried sclerotia of *S. sclerotiorum*. Only some of the constituents of sclerotia were found to be leached out. The increase in amounts of soluble carbohydrates leached with time of drying is consistent with the reports of some workers that sclerotia would be colonized by other microorganisms, which would presumably grow on leached carbohydrates and other metabolites.

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تحليل المواد الكربوهيدراتية المتسربة من سكلير وشيات الفطرة *سكلير وتينيا سكلير وشيورم* (لب) دوبارى خلال تعرضها للجفاف و الرطوبة

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عرضت سكلير وشيات الفطرة *سكلير وتينيا سكلير وشيورم* (لب) دوبارى لدرجات رطوبة نسبية مختلفة من الصفر إلى ١٠٠٪ ولفترات زمنية مختلفة وصلت إلى ١٦٨ ساعة. وقد لوحظ أن المواد الكربوهيدراتية تسربت بعد تبليل الاسكلير وشيات بالماء، وأن التسرب كان أكثر شيء عند الرطوبة النسبية صفر و ٣٣٪ وكان يزداد كلما زادت فترة التعريض لتلك الدرجات المنخفضة من الرطوبة النسبية. وقد حددت المواد الكربوهيدراتية المتسربة وكان ترتيب درجات تسربها على النحو التالى:

تريهالوز يليه المانيتول يليه الجلوكوز ثم الفركتوز وكان تسرب الاخير بكميات قليلة جدا.

وبتكرار تجفيف الاسكلير وشيات تم إعادة تبليلها فى الرطوبة النسبية صفر و ١٠٠٪ تسربت المواد الكربوهيدراتية بكميات أكبر، والكميات المتسربة وجد أنها تزداد بازدياد فترة تحضين الاسكلير وشيات فى الرطوبة النسبية وتنقص كلما زادت فترة تحضينها فى الرطوبة النسبية ١٠٠٪.