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Carbohydrate Accumulation and Utilisation Within and Between Rust Pustules in Leaves of Barley Infected with *Puccinia hordei*

D. M. Gwary[1], I. J. Tetlow[2] and J. F. Farrar[3]

[1]Department of Crop Science, University of Maiduguri, P. M. B. 1069, Maiduguri, Nigeria

[2]Department of Cell and Structural Biology, The Williamson Building, University of Manchester, Manchester, M13 9PL, U.K.

[3]School of Biological Sciences, University of Wales, Bangor, Gwynedd LL57 2UV, U.K.

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ABSTRACT: Carbon fluxes between and within rust pustules were studied in a barley infected with brown rust *Puccinia hordei*.. Analysis of the fluxes in whole infected leaves show that sucrose, fructose and glucose were reduced by 30%, 75% and 60% respectively in a typical diurnal cycle. Soluble sugars and starch accumulated rapidly during a photoperiod and were depleted during the following dark period, similar to the pattern and amount found in healthy leaves. Localised studies to within and between rust pustules revealed enhanced retension and accumulation of carbohydrates within the rust pustules. These carbohydrates (sucrose, starch and fructa) showed duirnal fluctuations both within and between pustules. A 3-fold increase in fructan in infected leaves was another striking observation following leaf infection. The paper discusses the source-sink relationship between the barley and rust biotrophic system.

Key words: Brown rust (*Puccinia hordei*); Biotrophic pathogen; Carbohydrate accumulation; Rusted barley; Diurnal cycle.

Introduction

The infection of barley by brown rust is a typical biotrophic parasitic relationship which has several consequences for the host plant. Among the major effects of such an interaction is the alteration of carbohydrate metabolism. Several studies have shown that infection by biotrophic pathogens can reduce photosynthesis and hence the sucrose available for translocation and other uses by the plant (Daly, 1976; Whipps and Lewis, 1981; Ahmed *et al*, 1983; Gwary, 1988; Tetlow, 1990; Wright *et al*, 1995). Such infections also lead to accumulation of carbohydrates in infected tissues as a result of either reduced tranlocation from infected sites (Siddiqui and Manners, 1971; Holligan *et al*, 1974; Ahmad *et al*, 1983) or due to import from healthy tissues of the same plant (Billet and Burnet, 1978). All previous studies of

carbohydrate metabolism in rust infected plants reported results that were based on measurements from heterogenously infected leaves with some parts of the leaf not infected. Similarly such previous studies lack the merit of showing diurnal changes of the effects of infection and consequently limits our understanding of the fluxes of carbohydrates in infected leaves especially when such fluxes have been well established for the healthy barley (Farrar and Farrar, 1984).
In this paper, in addition to showing the diurnal fluxes of carbohydrates in whole rust-infected leaves, we also show the localised diurnal fluctuation of these carbohydrates within and between pustules on infected leaves of barley. This includes the first report of fructan accumulation in this biotrophic system.

Materials and Methods

Plant growth and inoculation

Seedlings of *Hordeum distichum* (L.) Lam - cv Maris Mink were grown in John Innes No. 1 potting compost in a growth cabinet at 18°C, 0.61 Mpa vapour pressure deficit and a 16th day of 250 µmol, quanta M^2 S^2 of waveband 400-700nm, supplemented with tungsten filament bulbs. Second well expanded leaves were inoculated with urediniospores of *Puccinia hordei* Otth. as described previously (Owera *et al*, 1981) After inoculation the plants were kept back in the growth cabinet in the dark for 48h at 18°C under propagating lids to ensure high humidity necessary for infection. In this way, a density of 50 - 70 pustules cm^2 was achieved.

Extraction and assay of carbohydrates

Three replicates each consisting of 12 leaf discs from 2 to 3 second leaves were sampled. Discs 6cm in diameter were cut with a stainless steel punch, between 3 and 9 cm from the legule. To sample localised areas (rust pustules and areas between pustules) 3 replicates each consisting of 215 leaf discs, 1mm in diameter were taken from three second leaves. Plant materials were extracted twice in a total volume of 10cm3 of 95% ethanol at 80° and 60°C for the periods of 2 and 24 hours respectively. This extract is termed soluble carbohydrate extract. High molecular weight fructans (DP > 5) were extracted from plant reduces with 50 mol m^3 acetic acid/sodium acetate buffer pH 4.5. Materials left after ethanol extraction was ground in $5cm^3$ buffer with pistle and mortar. Ground samples were incubated at 25°C for 2h and filtered through a Whatman glass microfilter filter (GF/A, 2.1cm) on a sinter glass funnel under vacuum. The filterate was stored only for a short time at 4°C before being assayed. For the extraction of starch, the residue from fructan extraction was suspended in 5cm3 of acetic acid/sodium acetate buffer pH 4.5 to which 5 units of amyloglucosidase (from Rhizopus, Sigma) had been added. This enzymatic extraction was carried out for 24h at 40°C. The extract was similarly filtered and kept at 4°C prior to assaying.
Total carbohydrates in three different extracts were assayed by the phenol-sulphuric acid method of Dubois *et al* (1956).

Extraction and assay of acid invertase (AI)

One gram fresh weight of leaf was ground in a pestle and mortar in 4cm3 of extraction medium containing 500mol m^{-3} NaCl, 50 mol m^3 $Na_2HPO_4.2H_2O$, 25 mol m^{-3} KH_2PO_2, 5 mol m^{-3} sodium diethyldithiocarbamate. The resulting homogenate was centrifuged at 3000g for 10 minutes and the supernatant desalted by dialysing for 24h against 1 dm^3 of 500 mol m^{-3} NaCl. All procedures were carried out at 4°C.

For the assay of AI, the reaction mixture contained in a total volume of $400mm^3$; $100mm^3$ of tissue extract, 200 mm^3 of 100mol m^{-3} acetic acid/sodium acetate buffer pH 4.5 and 100 mm^3 of 500mol m^{-3} sucrose. The control reaction mixtures contained either boiled tissue extract or no sucrose. The reaction mixture was incubated at 30°C for 30 minutes. Reactions were stopped by adding 600 mm^3 of 500 mol m^{-3} Na_2HPO_4 and heating at 100°C for 3 minutes. Reducing sugars were measured using the ditrosalicyclic acid assay method (Miller, 1959). Invertase activity was expressed both as µmol sucrose hydrolysed h^{-1} g^{-1} fwt and µmol sucrose hydrolysed g^{-1} protein min^{-1}.

Measurement of protein

Protein content of extracts was determined using Coomassie brilliant blue G250 (Bradford, 1976). To 100 mm^3 extract, 5 cm^3 of Coomassie blue G250 reagent was added. After 15 min at room temperature, the absorbance was read at 595 nm. A calibration curve was prepared using 0-200 µg of bovine serum albumin.

Results

Diurnal variation of carbohydrates

Figure 1 shows a diurnal variation of soluble and insoluble carbohydrates in healthy and rust infected source leaves of barley. In healthy leaves, soluble carbohydrates (mainly sucrose) accumulated during the first 8 hours of the photoperiod and declined rapidly during the following 8-hour dark period. Lower amounts of starch and fructan accumulated and depleted in the same fashion. In rust-infected leaves although a similar pattern was observed, the amounts were lower but the fructan fraction increased by about 3-fold. Separation and quantification of the soluble sugars produced in both healthy and rusted leaves was carried out during the diurnal period (Figure 2). Although there was no marked pattern of diurnal activity among the hexoses, their concentration were reduced in infected leaves by about 30% for sucrose, 75% for glucose and 60% for fructose. Of the soluble sugar extract from healthy leaves, sucrose accounted for 83% of this, while glucose and fructose represented 9% and 8% respectively. Sucrose represented 92% of soluble sugar extracted from rusted leaves, glucose and fructose represented 3% and 5% of this fraction.

Table 1: Changes in activity of acid invertase in healthy and rust-infected barley leaves following inoculation with brown rust, *Puccinia hordei*.

Days after inoculation	Healthy leaves		Rust-infected leaves	
	µmol sucrose hydrolysed			
	g^{-1} fresh wt. h^{-1}	g^{-1} protein min^{-1}	g^{-1} fresh wt. h^{-1}	g^{-1} protein min^{-1}
2	61.96	126	53.08	110
4	48.16	96	51.11	104
6	44.21	92	53.08	106
8	28.44	58	55.06	109
10	21.05	52	83.15	179
12	21.84	46	60.28	143
16	16.61	40	50.82	104

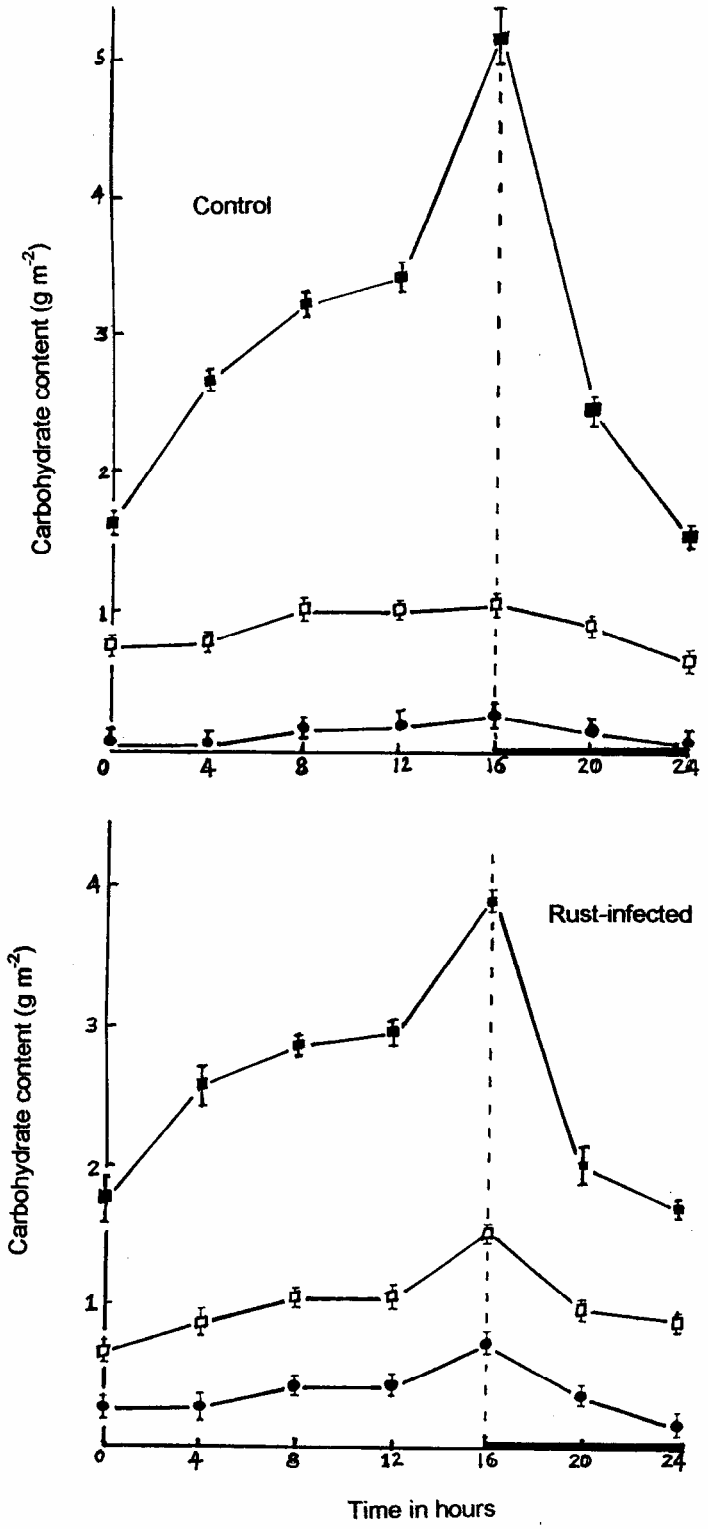

Figure 1. Diurnal changes in the mass of soluble sugars (■), fructans (●) and starch (□) in healthy and rust-infected leaf blades of Maris Mink barley cultivar on the day of fungal sporulation.. Leaves were sampled 8h into the 16h photoperiod. The dark period is shown by the solid bar on the x-axes.

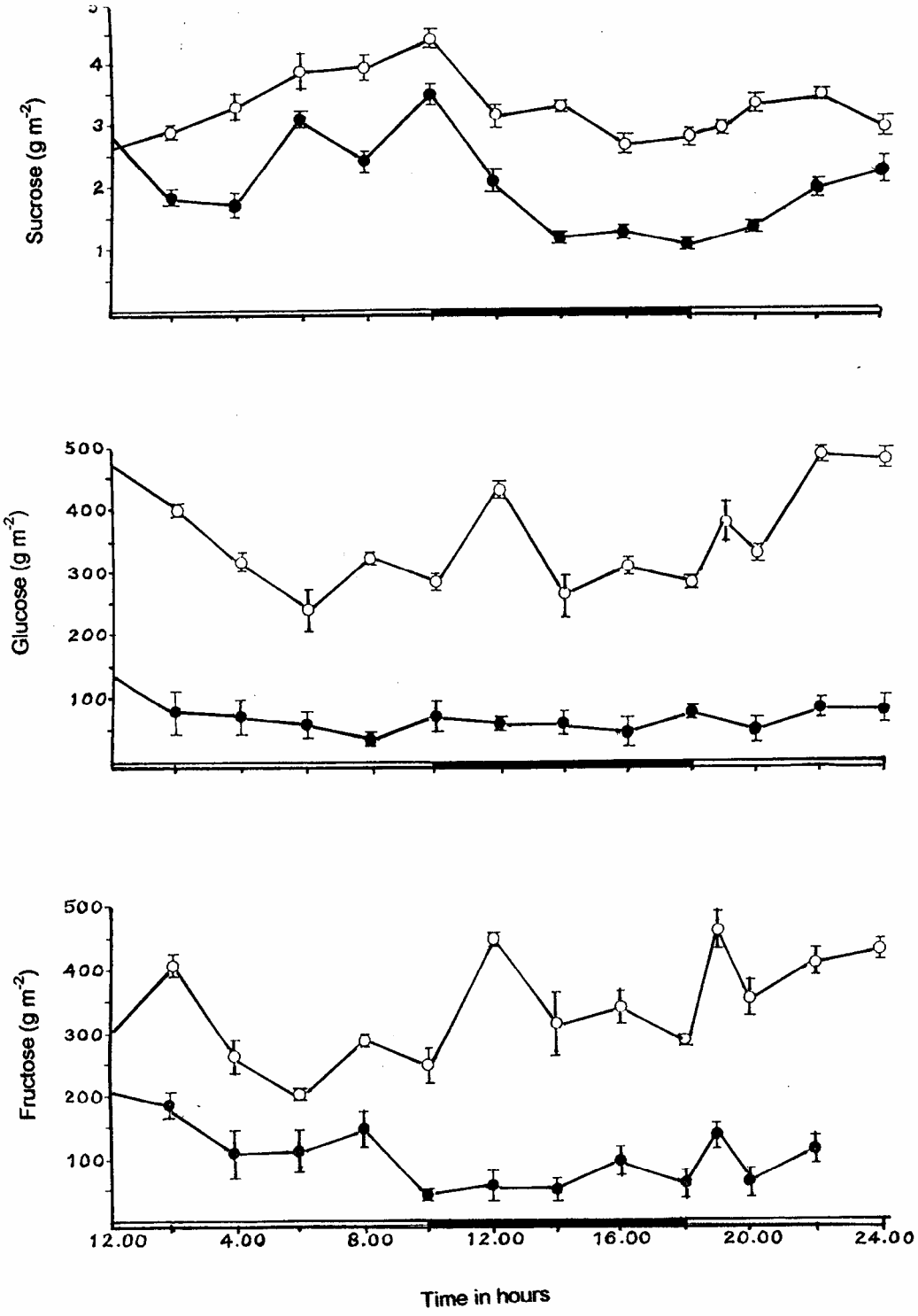

Figure 2.Diurnal changes in the soluble sugar content of healthy (○) and rusted (●) sporulating first leaves of Maris Mink barley. The dark period is shown is shown by the solid bar on the x-axes.

Diurnal variation of carbohydrates in and between pustules regions

Carbohydrates extracted from pustules and regions between pustules during sporulation also show diurnal variation. The amounts of soluble carbohydrates were significantly (P = 0.05) higher in pustules than between pustules or in comparative healthy controls (Figure 3). The amount of starch and fructan that were found in the pustule regions was also significantly (P = 0.05) higher than found between pustules or healthy controls. About 45% more of these fractions accumulated in pustules (Figure 4).

Changes in acid invertase (AI)

Rust infected source leaves of barley had increased ability to hydrolyse sucrose. Either measured as µmol sucrose hydrolysed in gram fresh weight per h^{-1} or in gram protein min^{-1}. AI in rusted leaves increased with progress of disease reaching its peak during sporulation (10 - 12 days after inoculation) which is the reverse situation in healthy leaves.

Discussion

The establishment of a compatible rust-barley interaction is a nutritional relationship in favour of the rust with great metabolic consequence for the host. During the early stages of infection of plant leaves when the fungal mass was small, nutrient flexes characteristic of healthy leaves were sufficient to sustain fungal growth. As fungal mass increased, continued fungal growth and reproduction is only possible by altering host metabolism. Studies here show how host carbohydrate are altered following infection. Fluxes of carbohydrate in diseased leaves cannot clearly be understood from mere measurements of changes in concentration. Variation of different carbohydrate fractions during a diurnal cycle at sporulation clearly shows that in both rusted and control leaves soluble sugars accumulated during the photoperiod and degraded rapidly to the initial concentration during the dark period. The result also shows that sucrose, fructose and glucose were reduced by 30%, 75% and 60% respectively at sporulation. Sucrose, the major constituent of the soluble carbohydrate is stored in the vacuoles during the photoperiod and mobilised during the darkness to maintain translocation (Farrar and Farrar, 1985). Gordon *et al* (1982) based on ^{14}C labelling reported that sucrose for export in the dark would initially be derived from the cytoplasm and would be replenished from vascular sucrose. Although the fungus has no direct access to the large store of vascular sucrose, there is now evidence for apoplastic sucrose pools which exchange with cytosolic pools via specific transport systems at the plasmalema (Huber and Moreland, 1981). Intercellular hyphae may have access to enough sugar from this source alone. With the fungus constituting an additional sink to the host, competing with the transport system of the host for these sugar, various ^{14}C translocation studies (owera *et al*, 1983; Gwary, 1988 and Tetlow, 1990) have demonstrated reduction in translocation from barley leaves infected with brown rust and proposed that the fall was largely due to a fall in amounts of sucrose in the vacuoles. This has led to the accumulation of different carbon compounds including fungal polyols at the site of infection as observed in this system. Holligan, McGee and Lewis (1974) have similarly observed α-glucans and ^{14}C incorporation into these compounds in leaves of Coltsfoot infected with *Puccinia poarum*.

In addition to linear accumulation of starch in infected leaves during the photoperiod, demobilisation during the dark period started only when sucrose and fructan had been reduced to a threshold concentration. Degradation of starch in the night has been suggested (Gordon *et al*, 1980a, b) to be controlled by the concentration of sucrose or other metabolites outside the chloroplast. This proposal seems to be compartable with the hypothesis of control of chloroplast metabolism by feedback from sink regions of the plant.

The numerous and small pustules of rust infections are not easy for individual manipulation therefore most previous investigations stopped at the level of the whole heterogenously infected leaves. Here we also show how carbohydrates localised within and between pustules change during the diel cycle. As much as 1.5 times more soluble sugars were found in pustules than in the regions between them. Although the mechanism involved in carbohydrate accumulation at infection sites is still to be resolved, reduced export

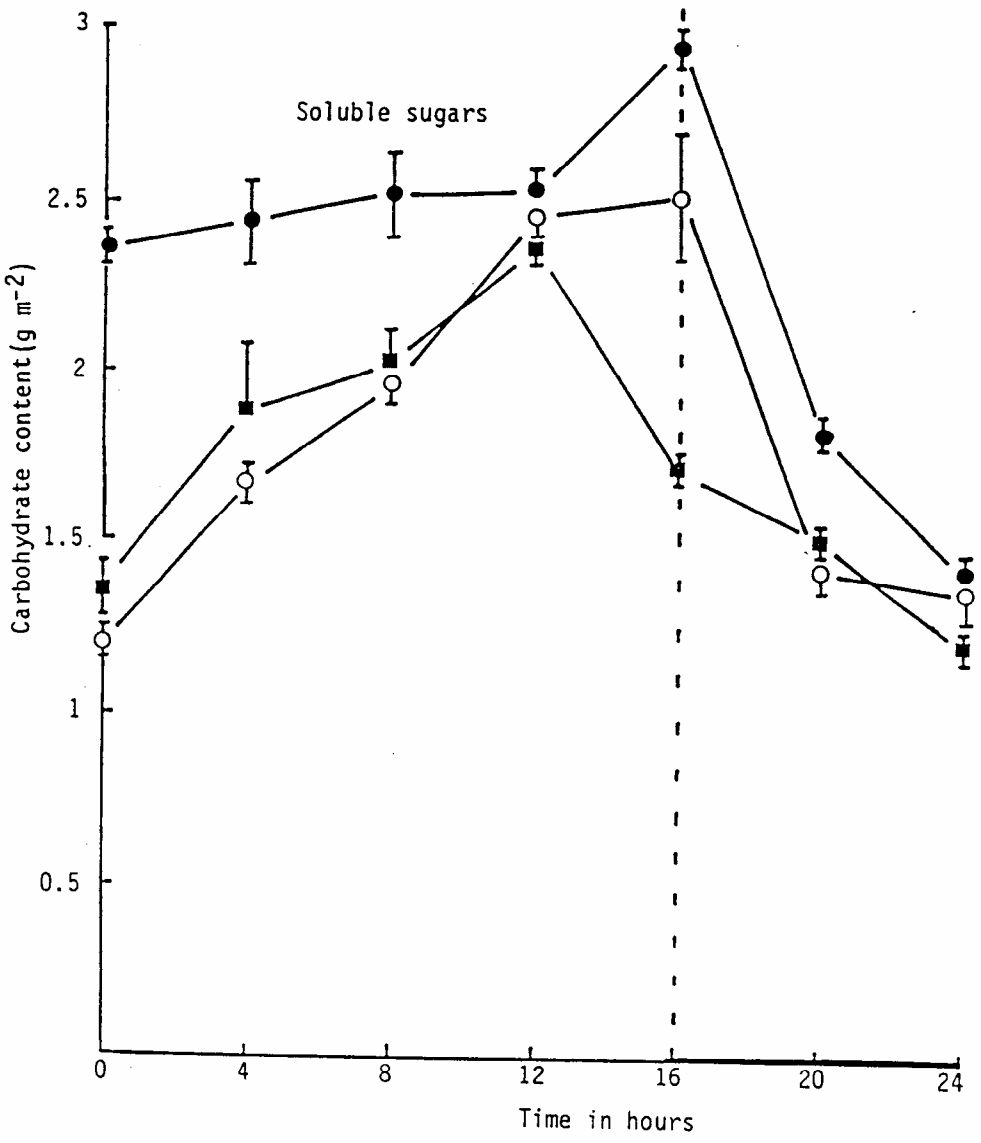

Figure 3. Soluble sugars over a diurnal cycle in healthy leaves (○), pustule regions (●) and between pustule regions (■) of rust-infected leaves on the first day of sporulation. The dark period is shown by the solid bar on the x-axis.

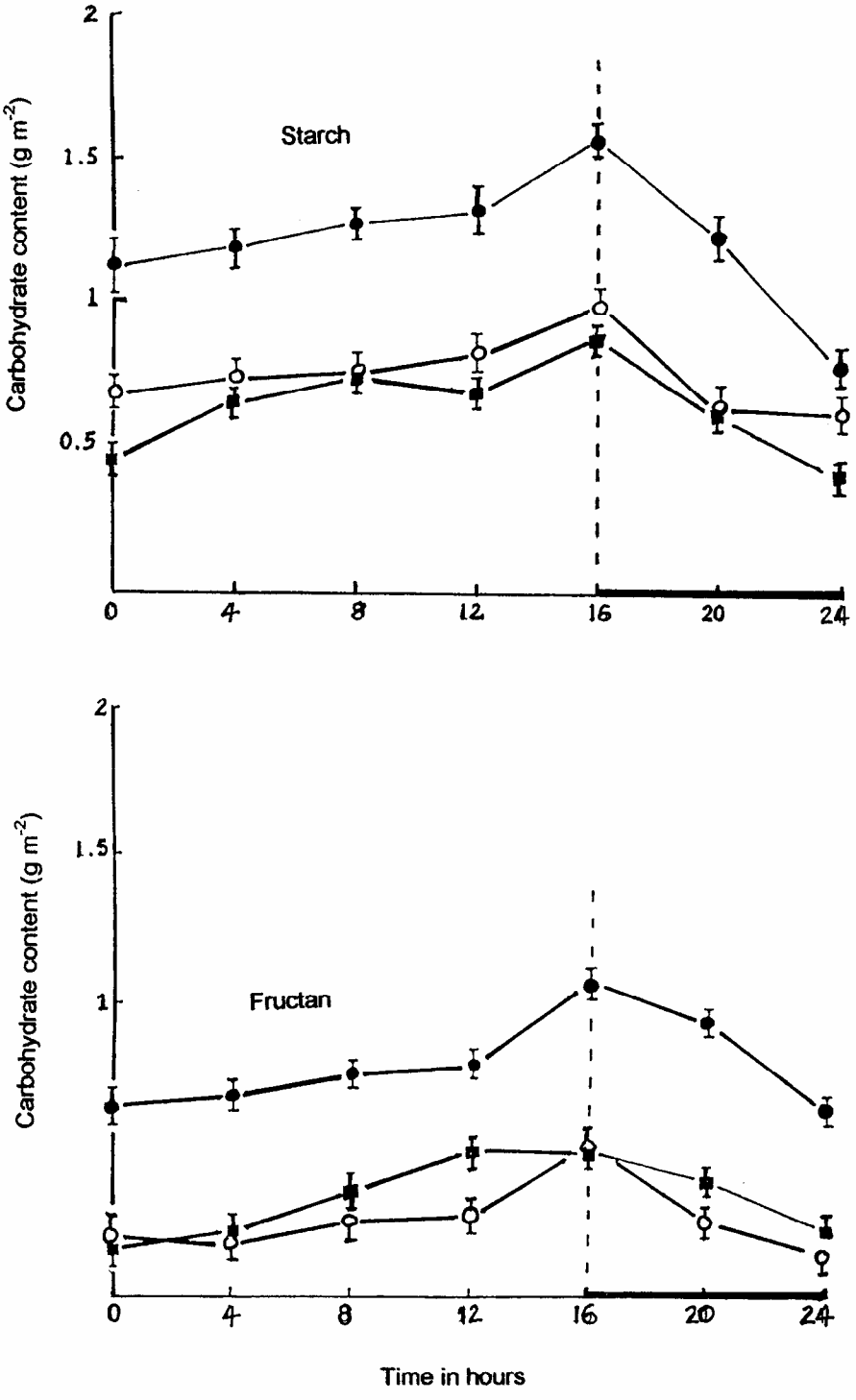

Figure 4. Starch and fructan over a diurnal cycle in healthy leaves (○), pustule regions (●) and between pustule regions (■) of rust-infected leaves on the day of sporulation. The dark period is shown by the solid bars on the x-axes.

and increased assimilate import from adjacent tissues as well as increased photosynthesis in these regions appear to account for this. Starch content in these regions also rose during the photoperiod and declined during the dark period. By histological staining, Bushnel (1970) also reported diel changes with starch in wheat infected with wheat stem rust accumulating in a zone 1-1.5mm beyond the margin of hyphal spread. Various hypotheses have been advanced to explain the mechanism of starch accumulation in infected leaves (Wang, 1961; MacDonald and Strobel, 1970) but it is now believed that AI plays a key role in providing hexoses for starch synthesis and for uptake by the biotrophic rust. Stimulation of AI at sporulation in diseased leaves reported here is consistent with the need to provide nutrition to the fungus through sucrose hydrolysis prior to uptake. The increased AI and the subsequent accumulation of carbohydrates at infection sites has remarkable effects on translocation of photosynthates from the source leaves to diatant sinks on the plant thereby affecting the carbon budget of the whole leaf (Gwary & Farrar, unpublished). The occurrence of fungal metabolites at sporulation was also reported elsewhere (Owera *et al*, 1983) and correlates with the increased AI activity and is consistent with the view that assimilate concentration gradient is established in favour of the pathogen as found in biotrophic associations (Smith *et al*, 1969). Our data does not show whether the induced AI is of host or fungal origin. However, it is reported in other disease systems as either mainly due to the host (Billett *et al*, 1977) or to the fungal pathogen (Callow *et al*, 1980).

Knowledge of the increased fructan concentration in diseased leaves has led to the question of the origin of such increases. Fructan extracted from both healthy and rusted leaf and run on thin layer chromatography (TLC) suggests that the fructan increase in diseased leaves was of host origin (Gwary, 1988(. Although it is not quite clear why fructan accumulates in infected leaves, it does appear as the increase is attributed to the build up of sucrose. Numerous studies have suggested that whenever the supply of assimilate by photosynthesis in temperate grasses and cereals exceeds the immediate demand for growth, excess carbon may be diverted into fructan synthesis (Pollock, 1984; Wagner *et al*, 1986; Cairns and Pollock, 1988). Such a change in carbohydrate balance of the leaf is apparent when the plants exposed to low temperature accumulated high concentration.

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