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The effect of fungal infection and nitrogen fertilization on the carbohydrate composition of *Rumex obtusifolius* leaves

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Abstract

The effect of infection by the rust fungus *Uromyces rumicis* and fertilization by different concentrations of nitrate or ammonium solutions on the concentrations of alcohol-soluble carbohydrates, fructans and starch in *Rumex obtusifolius* leaves was investigated. In leaves from healthy plants there was an increase in concentration of alcohol-soluble carbohydrates and fructans as the concentration of nitrate given was decreased, with the exception of nitrogen-stressed plants (those fed less than 10 mM l^{-1} nitrate) which had a lower concentration of alcohol-soluble carbohydrates than plants fed 10 mM l^{-1} nitrate. There was an increase in fructan and starch concentration in leaves as the concentration of ammonium solution given was decreased. The concentration of alcohol-soluble carbohydrates was reduced in infected leaves and increased in healthy leaves on infected plants, while the fructan concentration increased in infected leaves, compared to healthy leaves. The effects of infection were consistent over the range of nitrogen concentrations used, and thus were additive to the effects of fertilization. These results confirm the known effects of fertilization or fungal infection, singly, and indicate that, when combined, nitrogen deficiency and fungal infection may produce an additive stress on the plant.

Introduction

Low soil nutrient levels and infection by pathogenic fungi are two stress factors faced by plants that can result in altered plant metabolism and growth (Haynes and Goh, 1978; Chapin, 1980; Whipps and Lewis, 1981; Ayres, 1992). Given that the form and amount of nitrogen fertilization affects the severity of infection by many plant pathogenic fungi (Huber and Watson, 1974), we may expect an interaction between the physiological effects of nitrogen fertilization and fungal infection, with implications for the tolerance of multiple stresses by crop plants and weeds. However, this interaction has rarely been investigated.

In a previous experiment, we demonstrated that when fed nitrogen concentrations below 15 mM l^{-1} nitrate and 10 mM l^{-1} ammonium, growth of the weed *Rumex obtusifolius* L. was reduced, while high nitrate concentrations (above 25 mM l^{-1}) also led to reduced plant growth (Hatcher et al., 1997a). Total non-structural carbohydrate (NSC) concentration was affected both by infection of plants by the rust fungus *Uromyces rumicis* (Schum.) Wint. and nitrogen fertilization, and when combined the effect of infection was additive to that of nitrogen fertilization (Hatcher et al., 1997b). Infection by the rust alters partitioning of carbohydrate between NSC fractions in *R. obtusifolius* leaves (Hatcher et al., 1995b) and this also affects NSC storage in the roots (Hatcher, 1996). Host plant carbohydrate metabolism is particularly affected by fungal infection (Whipps and Lewis, 1981) and may also be altered by nutrient deficiency (Pollock and Cairns, 1991). We now report whether nitrogen deficiency or source also alters the partitioning to different NSC fractions in leaves of *R. obtusifolius*, and whether this also produces an effect additive to that produced by rust infection.

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Rumex obtusifolius is a short-lived perennial, widespread throughout the UK and Europe, and common on wasteground and permanent pasture (Cavers and Harper, 1964). *Uromyces rumicis* is a non-systemic rust fungus which has *Rumex* spp. as primary hosts and *Ranunculus ficaria* as the alternate host (Inman, 1971). Field infection of leaves of *Rumex* spp. usually occurs from August to October in northern UK.

Materials and methods

Plant rearing followed Hatcher et al. (1997a); only brief details are given below. *Rumex obtusifolius* seeds (from Herbiseed, Wokingham, UK) were germinated in a mixture of one part vermiculite (J. Arthur Bower's, Lincoln, UK) and one part (v/v) steam sterilized loam (Keith Singleton, Egremont, UK). When the first true leaf appeared, after about 10 days, seedlings were pricked out into 9-cm diameter pots containing 400 g of a mixture of three parts horticultural sand (Tarmac Roadstone (North West) Ltd, Buxton, UK) and two parts (v/v) steam sterilized loam. A similar soil : sand mixture was used by Melzer et al. (1984) for *R. obtusifolius*. All plants were grown in a constant environment (CE) room with a 16 h photoperiod with photosynthetically active radiation (PAR) of 500–700 μmol m^{-2} s^{-1} at plant height from 400 W metal halide lamps. The PAR above each plant was measured with a quantum photometer (model 550, T & J Crump, Rayleigh, UK), the treatments were applied randomly to the plants and the plants were re-randomized under the lights every week. Temperature was maintained at 20 ± 1 °C day and 16 ± 1.5 °C night.

Once pricked out into 9-cm pots, seedlings were watered on a daily rotation: day 1, deionised water; day 2, mineral solution; day 3, a nitrogen solution; day 4, deionised water, etc. The mineral solution was based on the ARC Letcombe nutrient solution (Paul and Ayres, 1986). The nitrogen-containing compounds were removed, and the composition was adjusted to retain the original balance between the other elements. The stock mineral mixture is given in Hatcher et al. (1997a), and the mixture was dissolved in 5-l deionised water to make a 75% strength ARC mineral solution.

At every third rotation of the solutions, sufficient mineral solution was given to allow 50 ml to flush through each pot. Apart from this, during the first week plants were given 30 ml of liquid daily, rising to 50 ml in week 2, 75 ml in week 3 and 100 ml in week 4. These amounts were found to give a constant ratio between supply and demand in trial experiments. The nitrogen solution contained 0, 1, 5, 10, 15, 25, 35 or 50 mM l^{-1} nitrate as sodium nitrate dissolved in deionised water. The experiment was repeated with 0, 5, 10 or 25 mM l^{-1} ammonium given as ammonium sulphate dissolved in deionised water (both ANALAR grade, BDH, Merck Ltd, Lutterworth, UK).

After 32 days growth in the 9-cm pots, 24 h after they had last been given the nitrogen solution, the plants were harvested. The harvest took place between 3 and 6 h into the light cycle, all leaves were removed from the plants, rinsed in deionised water to remove any residue from the nitrogen or mineral solutions, dried at 65 °C for 24 h and ground dry in a pestle and mortar.

Uromyces rumicis *infection*

Eight days before the harvest above, plants chosen at random from each of the nitrogen treatments were infected with *Uromyces rumicis* on all their leaves following our standard procedure (Hatcher et al., 1995a). Briefly, frozen uredospores were suspended in Fluorinert (FC-43, an inert fluorocarbon electronic liquid, 3 M Industrial Chemical Products Division, St Paul, Minnesota, USA) to produce a concentration of $2–5 \times 10^5$ spores cm^{-3}. This was sprayed onto leaves using a modeller's air brush to produce a uniform coverage of spores. The Fluorinert was allowed to evaporate and then the plants were moistened with water from a hand-held atomizer and enclosed in black plastic bags for 24 h in the CE room to facilitate infection. In the laboratory, *U. rumicis* usually showed first signs of infection after 5–6 days, and sporulated after 7 days. On the day of harvest, leaves were partitioned into those without infection and those with infection (this was not possible with the plants given 0 or 1 mM l^{-1} nitrate or 0 mM l^{-1} ammonium, because too little leaf material was present). The division between these categories was usually clear-cut; when it was not, leaves with less than 0.25 pustules cm^{-2} were placed in the uninfected category. Leaves were harvested and dried as above.

Chemical analysis

NSC's were determined using the methods of Farrar (1980). Samples of 25–35 mg leaf material were extracted sequentially as follows:

1. Boiled under reflux in 6 ml 95% ethanol for 1 h, rinsed, boiled with two further washes, the liquid

removed and made up to 10 ml. This fraction was the alcohol-soluble portion (Borland and Farrar, 1985);
2. The leaf samples were then incubated at 45 °C for 24 h in 10 ml acetate buffer (pH 4.5), and the liquid removed. This fraction corresponded to the fructan portion;
3. Finally, the leaf samples were incubated at 45 °C for 24 h in 10 ml acetate buffer (as above) with amyloglucosidase (from *Rhizopus* mould, Sigma Chemical Co., St. Louis, MO, USA) and this liquid removed. This fraction corresponded to the starch portion.

Carbohydrate concentrations in these extracts were determined colorimetrically by the phenol-sulphuric acid method (Dubois et al., 1956) immediately after the extracts had been produced. As we were interested in relative changes between fractions and not in absolute amounts of NSC, results from all the fractions were calibrated using sucrose standards. Chemicals used for all colorimetric assays were either of ANALAR grade (BDH, Merck Ltd, Lutterworth, UK) or ACS grade (Aldrich Chemical Co, Gillingham, UK), and two assay solutions were prepared from each sample. If the difference in absorbance between these solutions was greater than 2% the assay was repeated.

Statistical analysis

We recorded the proportion of the infected plant that was infected (see Hatcher et al. (1997a)) and thus by using the values from the chemical analysis of the infected and uninfected leaves of the infected plant, a mean value for all the leaves of the infected plant was obtained. Thus, three comparisons between leaves from the infected plant and the uninfected plant (all leaves from the infected plant, infected leaves only, uninfected leaves only) for infection and nitrogen concentration were carried out using two-factor ANOVA. This allowed us to determine whether *U. rumicis* infection had a constant effect over the range of nitrogen treatments (no significant interaction term) or whether the effect of infection varied with nitrogen fertilization (significant interaction term). For differences between healthy and infected leaves on the same plant, a linear regression ($y = a + bx$) of the difference between healthy and infected leaves against nitrogen concentration was calculated. The sign of a and the significance of the difference between a and $y = 0$ determined the difference between infected and uninfected leaves, and the significance of the slope b determined the significance of any interaction between infection and nitrogen concentration (Sokal and Rohlf, 1995).

Linear regression for each leaf class against nitrogen concentration was used to determine the presence of any significant linear relationship with increasing fertilizer concentration. Linear regression sufficed because although other equations or data transformations may have explained a greater proportion of the variance, we were interested only in ascertaining the presence of a relationship, and not its exact shape. Proportional data were arcsine square root transformed prior to analysis, and all analyses were carried out using MINITAB (v. 7.2).

Results

Plant growth and infection (all data are from Hatcher et al. (1997a))

Plants fed nitrate attained a maximum weight with 15 mM l^{-1} added nitrate, while a decrease in nitrate from 15 to 0 mM l^{-1} led to a decrease in leaf weight by 81% and total plant weight by 65%. Between 15 and 50 mM l^{-1} added nitrate there was a decrease in leaf weight of 28% and total plant weight of 36%. There was a decrease in leaf weight of 84% between plants fed 25 and 0 mM l^{-1} ammonium solution. Between 35 and 55% of the leaves (calculated from leaf weight) on infected plants fed either nitrate or ammonium solutions were classified as infected. The younger expanded leaves and unexpanded leaves generally remained uninfected. There was no linear trend in percentage of leaf weight infected with nitrate concentration, while the percentage leaf weight infected significantly increased with decreasing concentrations of ammonium fed to plants (Hatcher et al., 1997a).

Alcohol-soluble carbohydrates

Uninfected, nitrogen stressed plants (those fed 5, 1 or 0 mM l^{-1} nitrate) had a lower concentration of alcohol-soluble carbohydrates in leaves than those plants fed 10 mM l^{-1} nitrate, and as the nitrate concentration given increased from 10 to 50 mM l^{-1} there was a 15% decrease in concentration of alcohol-soluble carbohydrate (Figure 1A). There was no change in concentration of this NSC fraction over the range of ammonium concentrations used (Figure 2A, Table 1). In plants given nitrate solutions, infected leaves had a 15% lower concentration, and

Table 1. Effects of nitrate and ammonium fertilization, and infection by *Uromyces rumicis*, on the carbohydrate composition of *Rumex obtusifolius* leaves. R, all leaves from the infected plant; U, all leaves from the healthy plant; Rr, infected leaves on the infected plant; Ru, healthy leaves on the infected plant

Variable	Slope[1]				Analysis[2]			
	R	U	Rr	Ru	R vs U	Rr vs U	Ru vs U	Rr vs Ru
	Nitrate							
d.f.	1,78	1,78	1,58	1,58				
Alcohol-soluble	0	–	0	0	0	–	+	–
Fructans	–	–	–	–	+	0	+	0
Starch	0	0	0	0	0	0	0	0
	Ammonium							
d.f.	1,36	1,38	1,28	1,28				
Alcohol-soluble	0	0	0	0	+	0	+	–
Fructans	–	–	–	–	+	+	+	0
Starch	0	–	0	0	0	+	0	0

[1] +, –, 0, indicates a positive, negative, or no slope, respectively, from the linear regression of that variable against nitrogen concentration ($P < 0.05$).
[2] Results of two-factor ANOVA (the two leaf classes and nitrogen) are given for the first leaf class vs. the second, except Rr vs. Ru where linear regression was used, see text. +, –, 0, indicates the first class is significantly greater, less, or no different from the second leaf class, respectively. Significance level $P = 0.05$. There were no significant interaction terms.

healthy leaves on infected plants a 17% greater concentration of alcohol-soluble NSC, than leaves from healthy plants (Figure 1A). Healthy leaves from infected plants given ammonium solutions had 27% greater concentrations of alcohol-soluble NSC than leaves from healthy plants (Figure 2A). This effect was consistent over the range of nitrogen concentrations used for plants given either nitrate or ammonium solutions; thus the infection effects on alcohol-soluble NSC were additive to the effects of altering the nitrogen concentration given to plants (Table 1).

Fructans

In healthy plants there was an increase in fructan concentration of 64% as the concentration of nitrate solution given to plants decreased from 50 to 0 mM l^{-1} (Figure 1B), and an increase of 144% in concentrations of fructans as the concentration of the ammonium solution given to plants decreased from 25 to 0 mM l^{-1} (Figure 2B). Infected plants given ammonium solutions had a 58% greater fructan concentration in their infected leaves, and 27% greater concentration in their healthy leaves, compared to leaves from healthy plants (Figure 2B), while, in infected plants given nitrate solutions, the healthy leaves also increased in fructan concentration compared to leaves on healthy plants (Figure 1B). All infection effects were additive to the effects of the nitrogen given to plants (Table 1).

Starch

Starch content of leaves from healthy plants did not change as the concentration of nitrate solutions given decreased (Figure 1C), but there was a 133% increase in starch content in leaves from healthy plants as the concentration of ammonium solutions given decreased from 25 to 0 mM l^{-1} (Figure 2C). Starch content was unaffected by *U. rumicis* infection in nitrate-fed plants (Figure 1C), but was increased in infected leaves of plants given ammonium solutions, compared to leaves from healthy plants (Figure 2C, Table 1).

Discussion

Although all leaves on the infected plants were inoculated with *U. rumicis* spores, only some of the leaves became infected. These infected leaves had a lower alcohol-soluble NSC concentration than uninfected leaves on the same plant (Table 1). However, comparisons between healthy and infected leaves on the same plant should be made with care as infection by *U. rumicis* is age-related, with young, developing leaves tending to be resistant to *U. rumicis* infection (Hatcher et al., 1995a). Overall, the leaves from infected plants had higher fructan concentrations when

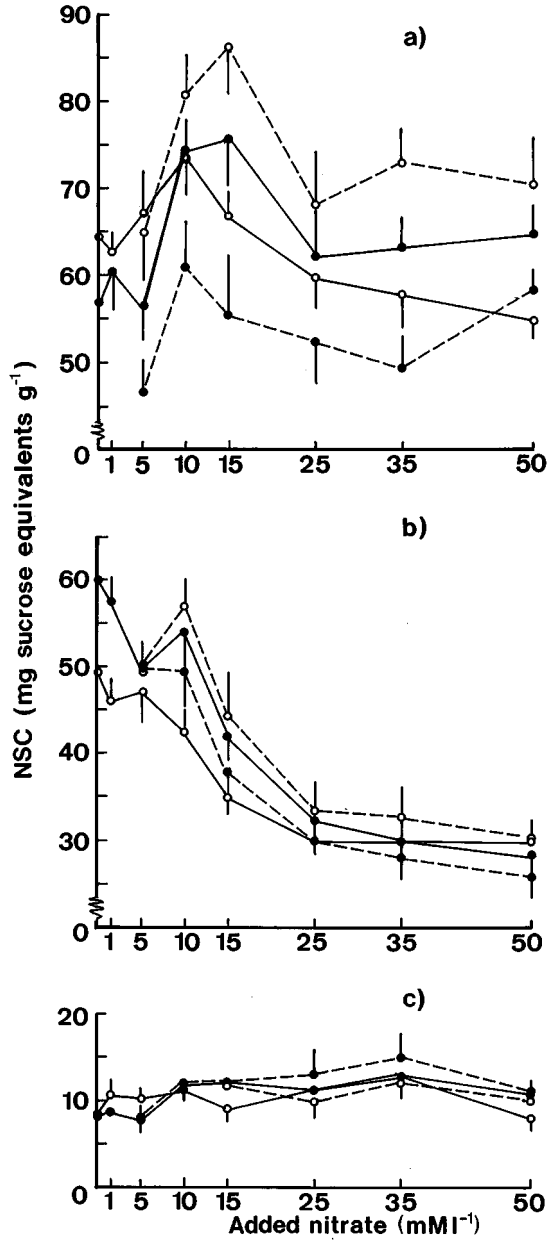

Figure 1. The effect of nitrate fertilization and infection by *Uromyces rumicis* on the concentration of leaf non-structural carbohydrate fractions in *Rumex obtusifolius*, A) alcohol-soluble carbohydrates, B) fructans, C) starch. Means ± SE given, although SE omitted sometimes for clarity, n = 10. ○——○, healthy plants (U); ●——●, whole infected plant (R); ○- - - - -○, healthy leaves from infected plant (Ru); ●- - - - -●, infected leaves from infected plant (Rr). See Table 1 for analysis.

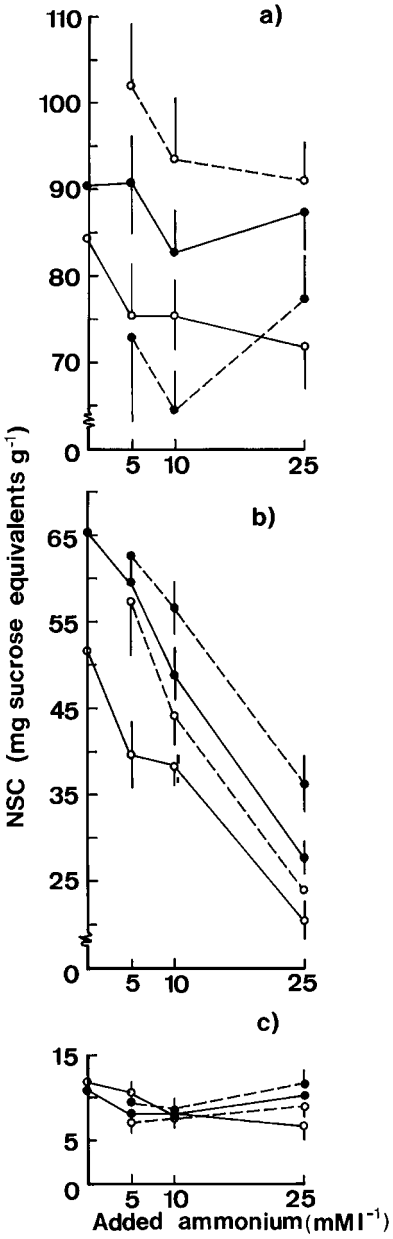

Figure 2. The effect of ammonium fertilization and infection by *Uromyces rumicis* on the concentration of leaf non-structural carbohydrate fractions in *Rumex obtusifolius*, A) alcohol-soluble carbohydrates, B) fructans, C) starch. Means ± SE given, although SE omitted sometimes for clarity. See Figure 1 for symbols. n = 10, except 0 mM l^{-1} R, where n = 8, see Table 1 for analysis.

fed nitrate or ammonium, and higher alcohol-soluble NSC concentrations when fed ammonium solutions, compared to leaves from healthy plants (Table 1). This is compatible with the overall increase in NSC concentration reported earlier for *R. obtusifolius* infected with *U. rumicis* (Hatcher et al., 1997b). In previous experiments, we have also observed a decreased partitioning of NSC to starch in the portion of uninfected leaf between the pustules, while the partitioning to alcohol-soluble carbohydrate and fructan fractions increased (Hatcher et al., 1995b). This is similar to that observed in several species (Whipps and Lewis, 1981; Farrar, 1989).

In our experiments it is possible that the amyloglucosidase extract (starch fraction) was contaminated by residual fructans (Borland and Farrar, 1985). Although any contamination would alter the absolute values recorded for the fractions, we believe that any contamination would be constant between treatments and thus would not alter our conclusions.

The leaves of healthy plants given nitrate solutions underwent an increase in concentration of alcohol-soluble NSC and fructan fractions, with no change in starch concentration as the concentration of nitrogen given to plants decreased and became limiting (Figure 1, Table 1). However, as the concentration of ammonium solutions given to healthy plants decreased, so both fructan and starch concentrations increased, while there was no change in alcohol-soluble carbohydrate concentrations (Table 1).

The effect of nitrogen fertilization on the concentration of different NSC fractions in leaves has received little study, although Archbold (1938, 1940) reported that both total leaf NSC content and fructan concentration increased with decreasing nitrogen fertilization in barley, leading to an alteration of the relative amounts of the different sugars present at different levels of nitrogen fertilization, results confirmed by our study. Likewise, the concentration of soluble sugars (mainly glucose and fructose) in leaves increased when *Arabidopsis thaliana* was given decreasing concentrations of ammonium nitrate solutions (Schulze et al., 1994). A similar result was found in tobacco, although leaf sucrose concentrations did not increase with a decrease in concentration of nitrogen fertilization (Fichtner et al., 1993).

Accumulation of starch is likely to occur when low levels of nitrogen limit metabolism of photosynthesized sugars for protein synthesis (Herold, 1984; Schulze et al., 1994; Mooney et al., 1995). As nitrogen fertilization increases and nitrogen is no longer limiting for plant growth, carbohydrates are remobilised. Fructans form an accessible reserve carbohydrate store (Pollock and Cairns, 1991) and are the most heavily depleted (Figures 1, 2). In healthy *R. obtusifolius* plants given nitrate, further depletion of the leaf alcohol-soluble NSC occurs (Figure 1) while with ammonium fertilization depletion of the leaf starch reserves takes place instead (Figure 2).

Differences in other aspects of *R. obtusifolius* metabolism between ammonium and nitrate fertilization have been observed (Melzer et al., 1984; Hatcher et al., 1997a, b). These differences, and those reported in these experiments, may reflect the different physiological responses of the plant to these ions; since high levels of ammonium ions in plants become toxic, these ions must be used immediately to synthesize organic molecules, leading to depletion of NSC reserves (Figure 2). Nitrate is not so toxic, may be accumulated, and must be reduced before it is assimilated; thus it affects NSC levels less (Haynes and Goh, 1978).

This study has provided the first evidence that the effect of biotrophic fungal infection on leaf carbohydrate composition acts in an additive manner to the effect of nitrogen fertilization by both nitrate and ammonium solutions. This additive interaction is perhaps surprising since there was a significant decrease in pustule density and in percentage sporulation eight days after inoculation with increasing concentration of nitrate given to plants, but not with increasing ammonium (Hatcher et al., 1997a). However, this additive interaction is similar to the effect of nitrogen fertilization on oxalic acid concentration in *R. obtusifolius* (Hatcher et al., 1997b) and suggests that in *R. obtusifolius* given nitrate solutions, pro rata, each *U. rumicis* pustule must have been having a greater effect on plant metabolism as the concentration of nitrate fed to the plant was increased.

A diseased leaf typically photosynthesizes less than a healthy one yet, in addition to the normal use of photosynthate, these leaves have increased respiration compared to a healthy leaf due to growth of the pathogen and defence responses. This leads to a smaller proportion of NSC available for export (Farrar, 1992). Therefore, a biotrophic fungal infection, coupled with the plant's use of NSC to detoxify the effects of fertilization by ammonium, must have a significant effect on the amount of NSC available to be mobilized to the roots. Since root weight correlates with regrowth ability in *R. obtusifolius* (Monaco and Cumbo, 1972; Hatcher, 1996), this should have consequent effects on the regrowth ability of this weed.

The experiments reported here have highlighted the possibly important role of fructans in *R. obtusifolius*, as this is the NSC fraction that undergoes the greatest change with both nitrogen stress and fungal infection. The research also highlights how little is known about the role of these sugars in plant stress physiology, and especially in response to multiple stresses. Further research needs to clarify these points for dicotyledons as well as for monocotyledons.

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