Can plant sugars mediate the effect of nitrogen fertilization on lettuce susceptibility to two necrotrophic pathogens: Botrytis cinerea and Sclerotinia sclerotiorum?

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Can plant sugars mediate the effect of nitrogen fertilization on lettuce susceptibility to two necrotrophic pathogens: *Botrytis cinerea* and *Sclerotinia sclerotiorum*?

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Abstract

Aims. Nitrogen (N) fertilization is known to modify a plant's susceptibility to necrotrophic diseases. However, the effect of N nutrition on defence is not well known. It was hypothesized that not only molecules related to the N metabolism but also main sugars could mediate the effect of plant fertilization on its susceptibility to pathogens.

Methods. Two necrotrophic fungi, *Botrytis cinerea* and *Sclerotinia sclerotiorum* were inoculated on leaves of lettuce plants grown at 5 nitrate (NO_3^-) fertilization levels, in three independent experiments. Variations in plant composition at the time of inoculation were linked to the size of lesions observed after 5-6 days.

Results. Both diseases were favoured by high NO_3^- fertilization. However, the highest disease levels were not found in the same experiment for *B. cinerea* and *S. sclerotiorum*. Among the components measured, NO_3^- and sucrose (SUC) were positively and negatively correlated to the two diseases in the three experiments, but the relationship between SUC and lesion size was more significant for *S. sclerotiorum*. Water content, N and total carbon (C) were also significantly correlated to the diseases, but the relationships were less straightforward. The ratios of SUC over total sugars and fructose (FRU) over total sugars fitted, very closely for *S. sclerotiorum*, a negative and positive exponential relationship respectively with lesion size. Absolute or relative glucose (GLU) levels were not linked to the diseases.

Conclusions. Plant metabolic modifications induced by variations of N availability conferred the plant variable defence ability, which seemed, at least for *S. sclerotiorum*, mainly mediated by variations in host SUC and FRU levels. The generalization of these findings to other species would be of interest.

Keywords: *Lactuca sativa* L; plant disease; pathogenic fungi; sucrose; fructose.

Introduction

Botrytis cinerea Pers. Fr. and *Sclerotinia sclerotiorum* (Lib.) de Bary are two phylogenetically close necrotrophic ascomycetes (Amselem et al. 2011), sharing many common characteristics in their development and pathogenicity. Both feed and grow on previously killed plant host cells (Williamson 2007; Bolton et al. 2006). Both fungi can infect a very wide range of plant species and, to kill their hosts, secrete a variety of compounds including oxalic acid, cell-wall degrading enzymes, peptidases, and a pool of toxic metabolites. These compounds allow the pathogen to modify the host redox status, perturb its defence, alter the cell integrity and macerate the plant tissues (Alghisi and Favaron 1995; Riou et al. 1991; Godoy et al. 1990). Common patterns of host defence responses upon infection by the two necrotrophs have also been demonstrated (El Oirdi et al. 2011; Glazebrook 2005; Oliver and Solomon 2010; Robert-Seilaniantz et al. 2011). They lead, on the one hand, to the local production of reactive oxygen species which trigger the hypersensitive response and ultimately programmed cell death. On the other hand, the plant activates the secretion of fungal enzyme antagonists, including inhibitors of cell wall degrading enzymes (Juge 2006), anti-fungal secondary metabolites, and cell wall strengthening molecules (Stotz et al. 2011; Kliebenstein 2004; van Baarlen et al. 2004). Whatever the similarities in infection strategies and stimulation of plant defences, the secretome diversity of both fungi allows them to adapt to their broad host range (Amselem et al. 2011; Choquer et al. 2007).Strain-specific aggressiveness, possibly linked to toxin synthesis and/or detoxification of plant defence metabolites, has been frequently reported (Choquer et al. 2007; Siewers et al. 2005). Although they are frequently associated in the field, especially in lettuce cropping systems, only a few comparative studies of symptom development of these two fungi have been conducted.

Upon infection, a massive change in the host's genome expression is triggered (Katagiri 2004), which coincides with an enhanced use of plant primary metabolites towards defence-oriented pathways and the use of energy and C and N skeletons for defence (Bolton 2009; Berger et al. 2007). Negative or positive correlations between plant sugar status and susceptibility to fungi have long been noticed (Levy and Cohen 1984; Horsfall and Dimond 1957), leading to the concept of low or high-sugar pathogens (Horsfall and Dimond 1957). Several studies have reported a decrease in photosynthetic activity and an increase in leaf cell-wall invertase activity after infections by either biotrophic or necrotrophic fungi

(Berger et al. 2004; Scharte et al. 2005; Kocal et al. 2008; Fotopoulos et al. 2003). A rise in the concentration of soluble sugars can induce the synthesis of defence related molecules (Johnson and Ryan 1990; Ehness et al. 1997; Morkunas et al. 2011) and the onset of the hypersensitive reaction (Essmann et al. 2008). However, hexoses are also thought to be the principal source of C for the pathogen, and both *B. cinerea* and *S. sclerotiorum* secrete their own invertases to exploit the host sugar content (Jobic et al. 2007; Dulermo et al. 2009). Along with the host carbohydrate metabolism, the plant N status largely determines the outcome of an infection.. However the relationship between N availability and plant constitutive or induced immunity is not straightforward (Walters and Bingham 2007). Generally, higher concentrations of secondary metabolites active in plant defence, notably polyphenols, are found in plants grown at low N availability (Stout et al. 1998; Le Bot et al. 2009; Lou and Baldwin 2004). Nevertheless, the accumulation of secondary metabolites in N-deficient plants is not readily linked with decreased susceptibility to necrotrophs such as *B. cinerea* (van Baarlen et al. 2004) or *Alternaria solani* (Mittelstrass et al. 2006). Indeed, low N availability is known to limit the plant contents of amino acids and proteins, which could include constitutive or inducible pathogenesis-related proteins (Dietrich et al. 2004). Also, the influence of the host N status on its ability to regulate nitric oxide synthesis, which has recently been reported as an important component of plant defence against *B. cinerea* and *S. sclerotiorum* (Asai and Yoshioka 2009; Perchepied et al. 2010), is not known. Furthermore, as for sugars, fungal pathogens retrieve N from their hosts, presumably with amino-acids as preferential source (Solomon et al. 2003). Whether the N content of the host can be limiting for pathogen growth is still a matter of debate (Bolton and Thomma 2008; Solomon et al. 2003). The availability of plant-based N compounds might however depend on the plant N status and the speed of fungal expansion, as suggested from disease progress observations (Lecompte et al. 2010; Newton and Guy 1998). As a result of these multiple processes, the effects of host N content on its susceptibility to necrotrophic pathogens are variable, either positive, negative, or neutral (Hoffland et al. 2000; Lecompte et al. 2010; Long et al. 2000; Huber and Thompson 2007). Based on the above evidence that the mobilization of plant primary metabolism can markedly affect the outcome of the host-pathogen relationship, and given the interdependence of carbon (C) and N metabolism (Nunes-Nesi et al. 2010), it is surprising that very few studies have examined the combined effect of C and N availability on fungal disease development. An

experiment with two levels of N at ambient and elevated CO_2 concluded that high N and CO_2 reduced epidemics caused by *Cercospora* sp. on *Solidago rigida* (Strengbom and Reich 2006). However, in this work, the various C and N-based metabolites were not analysed with regard to disease severity. We hypothesized that the effect of N nutrition on epidemic severity could be partially due to modifications of the plant C status, especially its sugar content, and to its impact on triggering or fueling host immunity. The questions we addressed were: (i) which mathematical function(s) gave a consistent relationship between lettuce NO_3^-fertilization and the symptoms caused by *B. cinerea* and *S. sclerotiorum* ; (ii) was the fertilization effect on each disease related to the plant N status or to the main plant sugars? We report an experimental study on lettuce grown at five N-supply levels, during three different seasons in a greenhouse, leading to marked variations in C and N contents. We assessed and compared the severity of disease caused by *B. cinerea* and *S. sclerotiorum* and related the development of lesions with total C, total N, NO_3^-, sugars and other nutrients in plant tissues. The relative proportion of sucrose (SUC) and fructose (FRU) were closely correlated, negatively and positively respectively with lettuce susceptibility to *S. sclerotiorum*, while infections with *B. cinerea* were correlated most closely with plant $NO_3^-$and total N.

Materials and methods

Experimental design

Three batches of 200 lettuce plants were grown from March 31st to May 30th 2009 (experiment E1), September 7th 2009 to November 11th 2009 (experiment E2) and November 16th 2009 to January 14th 2010 (experiment E3). . For all experiments, seeds of cultivar Faustina (Rijk Zwaan) were sown in 1 cm^3 rockwool cubes in a nursery greenhouse. Ten days after sowing, the cubes, each containing one seedling were transferred to 10 x 10 x 6 cm rockwool blocks (Grodan, Roermonds, The Netherlands) and placed in a second greenhouse dedicated to the experiments. Plants were then grown for approximately one month and irrigated twice a day with a standard commercial nutrient solution (Plantain, Duclos international, Lunel, France). After that period, the rockwool blocks (bearing plants with 3-4 developed leaves) were transferred on to the top of 2 L pots filled with a mixture (1:1 V/V) of

vermiculite and pozzalana (inert crushed volcanic rock) to start the nutrition treatments. The experimental design was monofactorial, with four randomized blocks of 10 plants per treatment. Five NO_3^- concentrations were tested in the fertilization solution: 0.5, 2, 5, 10 and 20 mmol.L^{-1} NO_3 (abbreviated as mM in the rest of this paper). The composition of the five solutions, made up from simple salts, is given in Table1. At NO_3^- concentrations below 10 mM, NO_3^- ions were replaced by sulphates, by the use of potassium sulphate instead of potassium nitrate. At the highest nitrate level (20 mM), the concentration of potassium nitrate was doubled. We considered that potassium nitrate was better than other NO_3^- salts to achieve the doubling of NO_3^- concentration in solution. The concentrations in solution of other macronutrients (calcium, magnesium and phosphorus) and trace elements (B, Fe, Cu, Mn) were kept constant. The ionic charge was neutral in all solutions when accounting for Cl^- and HCO_3^- ions already present in the irrigation water, but the electrical conductivity was 40% higher at the highest NO_3^- level (20 mM). The pH of each solution was adjusted to 6.5 by adding H_2SO_4. Nutrient solutions were supplied via a fertigation network with an individual dripper into each pot. Three pots in an additional block with a 20 mM fertigation were continuously weighted to estimate daily water losses, which were replaced by fertigation pulses, up to six times a day, depending on the external radiation and the crop growth stage. All plants, whatever the nutrient solution, received the same amount of water. Excess water was lost in drainage. Plants were grown for three additional weeks with these different NO_3^- concentrations in the fertigation solution. At the end of the period, the 70 day old plants were either used for inoculation with *B. cinerea* or *S. sclerotiorum* or for nutrient content assessment. Depending on the season, the greenhouse was either cooled or heated. The incoming radiation gradually increased from 1000 to 2300 J.cm^{-2} in E1, decreased from 1800 to 750 J.cm^{-2} in E2 and remained around 500 J.cm^{-2} in E3. Daily average air temperatures increased from 17 to 27°C in E1, fluctuated between 15 and 25°C in E2 and decreased from 17 to 10°C in E3.

Analysis of plant components

Five plants were randomly selected from each N nutrition regime in the greenhouse for determination of plant fresh and dry weight and for analysis of different minerals and primary metabolites just before

inoculation. The plants were harvested at similar times of day in the three experiments, all around 8 am. The fresh and dry mass of the aerial parts were measured, while roots were discarded. A subsample of 3 or 4 leaves from the intermediate crown were kept for plant analysis. Immediately after sampling, the leaves were placed in liquid N. Ten plant components (primary metabolites or elementary elements) were measured, namely: total N (N), total C (C), nitrate (NO_3^-), glucose (GLU), fructose (FRU), sucrose (SUC), phosphorous (P), potassium (K), magnesium (Mg) and calcium (Ca). Sub samples of dry material were ground, calcined at 400 °C for 12 h and then mineralized in boiling HNO_3. The K, Ca and Mg contents were measured with an atomic spectrometer (Varian A220), and the P content with a spectrophotometer (Perkin-Elmer Lambda 2). Total N and C were measured with a gas analyzer (Thermo Finnigan EA1112), and NO_3^- with a N03/N02 analyzer (5000 FIAstar). Soluble sugars were determined with an enzymatic method in a microplate reader, as proposed by Gomez *et al.*(2007). Hexoses (FRU + GLU) and total sugars (FRU + GLU + SUC) were computed from these analyses, as well as the C/N ratio.

Inoculation and disease assessment

Six strains of *B. cinerea* and one strain of *S. sclerotiorum* were used in this study**.** The strains of *B. cinerea* (BC1, BC43, BC44, BC21, BC84, NHPm4) where chosen for their contrasted aggressiveness on tomato (Lecompte et al. 2010), but without preliminary information on their aggressiveness on lettuce. For each strain, the inoculum was produced on potato dextrose agar medium (39 g L^{-1} Difco, Detroit, USA) in a growth chamber (Heliofroid, Le Beausset, France) at 21 °C with a 14h photoperiod. For each strain of *B. cinerea* and *S. sclerotiorum*, one set of five plants per NO_3^- treatment (35 plants in total) was inoculated. On each of these 70 day old plants , three leaves of the middle crown were inoculated. A 5 mm diameter mycelial disk, excised from a 3-day old colony, was placed in the centre of each leaf. The leaf inoculations were made on intact plants. Following inoculation, the plants were placed in a growth chamber and incubated for seven days in conditions conducive to disease development (21 °C, RH above 85 %, with a 14h photoperiod). During this period, the plants were irrigated manually twice a day, using the same fertilization solutions as those used before inoculation.

Very few of the 1575 observed leaves failed to develop lesions, and 0 values were discarded. The size of the lesions was assessed 5 and 6 days after inoculation for *S. sclerotiorum* and *B. cinerea*, respectively. At these dates, the lesions had not yet covered the whole leaf surface. Each inoculated leaf was detached and photographed over a blue background. The image analysis software Assess 2.0 (APS Press, St Paul, MN, USA) was used to quantify the leaf area (mm^2) and the lesion size (mm^2).

Data analysis

For the 5 NO_3^- nutrition levels and 3 experimental replicates, 1350 and 225 observations were analysed for *B. cinerea* and *S. sclerotiorum* respectively. The SAS statistical package was used for the data analysis. The first step was to analyse by general linear models (GLM) the effect of the three (or two) experimental factors related to *Botrytis cinerea* (or *Sclerotinia sclerotiorum*) lesion development: "NO_3^- concentration in solution", "experiment" , "*B. cinerea* strain" (for *B. cinerea* only), and their interactions. Significant differences among treatments were determined by the Neuman-Keuls test. Secondly, the type of mathematical function relating NO_3^- concentration with disease severity was assessed. Six types of functions were tested, whose equations are as follows, where L is the lesion size (mm^2), R the regressor, and a, b and c are parameters:

Linear: $L = a + b * R$

Exponential: $L = a * \exp(b * R)$

Power: $L = a + b * R^c$

Logistics: $L = \frac{a}{\left(1+\left(\frac{a}{b}-1\right)*exp(-c*R)\right)}$

Michaelis-Menten: $L = a * \frac{R}{b+R}$

Hill: $L = a * \left(\frac{R^b}{c^b+R^b}\right)$

The Gauss-Newton method was used to estimate parameter values, using the NLIN procedure of the SAS package (with 449 degrees of freedom (df) for each regression model for *B. cinerea* and 74 for *S.*

sclerotiorum). For each disease, the model minimizing the mean square error of prediction (MSEP), was chosen.

Thirdly, the plant components linked to disease variability were investigated, using the fresh weight data. Variables whose distributions were highly skewed and significantly different from a normal distribution were log-transformed. As plant analyses and leaf susceptibility assays were not done on the same plants, the regressions had to be performed on mean values, providing 14 df for each model with the pooled data (for global multiple regression), and 4 df for regressions on individual plant components in each experiment. This means that for each N level in each experiment the mean lesion area observed on leaves was related to the mean nutrient content of 5 other plants that received the same nutrient solution. However as the trend of the relationship between plant nutrient content and susceptibility was similar for all strains, the *B. cinerea* inter-strain variability was not considered in this part of the analysis and the overall mean from 90 measurements was used: 3 leaves x 6 strains x 5 plants. Fifteen measurements (3 leaves x 1 strain x 5 plants) were used for *S. sclerotiorum*. In the linear regressions between lesion areas and individual plant component for each experiment, only those components which were, in the three experiments, highly correlated ($r^2 > 0.7$) to disease severity were retained. Among these components, several showed high auto-correlation levels. Repeated GLM analysis – with the pooled data from the three experiments - between disease severity and plant components were thus performed, with all possible combinations of variable orders, and the type I sums of squares (SS) were analysed. When a significant F-value for a given variable was obtained whatever the order of its entry in the model, it was assumed to be independent of other variables.

On a fourth step, the same six functions described above were tested to relate the influence of the selected plant components on both diseases.

was tested.

Results

Lettuce growth, primary metabolites and ions contents

Leaf area increased asymptotically with NO_3^- concentration in the fertigation solution ($p<0.0001$,Fig. 1). Significant differences were found between experiments ($p<0.0001$), but in all cases no further increase was observed for concentrations beyond 10 mM. NO_3^- concentration also had a significant effect on fresh weight (g), dry weight (g) and plant water content (g water g^{-1} dm), following the same pattern, with no increase beyond 10 mM NO_3^-. Water content almost doubled with the treatments, from 8.5 g g^{-1} at 0.5 mM NO_3^- to around 17g g^{-1} at 10 and 20 mM NO_3^-, and was closely correlated with total N content (data not shown). NO_3^- nutrition had a very significant effect on the ten plant components measured, either on a fresh or on a dry weight basis (Table 2, data shown only on a fresh weight basis). All elements except Ca showed significant variability between experiments, and the "NO_3^- " x "experiment" interactions were also always significant. Almost no NO_3^- was found in plants grown at very low NO_3^- concentrations, but the plant NO_3^- content increased exponentially with increasing NO_3^- fertilization regimes. At the 10 and 20 mM NO_3^- regimes, plant NO_3^- content was much higher in E1, with nitrate N accounting for 15% and 30% of total N respectively as compared to 5-6% (at 10 mM) and 7-8% (at 20 mM) in the two other experiments. Similarly to plant NO_3^- , total N increased up to 20 mM NO_3^-. In contrast with plant NO_3^- , N accumulation was lower in E1. The highest SUC levels were found at 2 mM NO_3^- and SUC decreased at higher NO_3^- regimes, while GLU and FRU contents increased up to 5 mM NO_3^- (Table 2). The variation in the plant total sugar content (SUC+GLU+FRU) with the fertilization regime was comparable in the three experiments, with an increase from 0.5 to 5 mM NO_3^- and lower values at higher NO_3^- regimes (Fig. 2a). However the proportion of SUC in the sugar pool decreased sharply with NO_3^- nutrition up to 10mM, and appeared fairly different from one experiment to another, with a much higher proportion of SUC in E3 (Fig. 2b). Total C, P, K, Ca and Mg contents decreased, on a fresh weight basis, with increasing NO_3^- concentration in the nutrient solution (Table 2). The plant C content decreased exponentially with the NO_3^- regime and was highest in E3. In that experiment, on a dry weight basis, the C content did not change with NO_3^- regime, while it decreased strongly with NO_3^- nutrition in E1. On a dry weight basis, P and K plant content increased with increasing NO_3^- concentrations (data not shown). The increased K^+ concentration in nutrient solutions at 20 mM NO_3^- did not have a systematic effect on plant K accumulation in this regime, as plant K contents were not higher at 20 mM NO_3^- compared to those at 10 mM NO_3^-, in the E2 and E3 experiments. With pooled

data from the three experiments, many of the plant components appeared significantly correlated. The most highly correlated components (on a fresh weight basis), were GLU and FRU (r=0.95; 0.75; 0.76 in resp. experiments E1,E 2 and E3 respectively), Ca and C (r=0.92; 0.93; 0.80), SUC and C (r=0.93; 0.79; 0.81) and Mg and K (r=0.5; 0.79; 0.89). A strong negative correlation was also found between SUC and NO_3^-plant content (r=--0.75; -0.91; -0.87). On pooled data, the plant water content was highly correlated with the C content (r=-0.96) and SUC content (r=-0.81). SUC was highly negatively correlated with FRU (r=-0.64) but only slightly with GLU (r=-0.39).

Disease development at different N nutrition levels

Overall lesion sizes were different for the two fungal pathogens, and for a given pathogen, they were significantly different from one experiment to another (Fig. 3a). Lesion size was greater in E1 for *B. cinerea*. Lesions caused by *S. sclerotiorum* appeared more severe in E2, whereas in that experiment lesions caused by *B. cinerea* were minimal. Necrotic lesions developed usually as an ovoid shape, being slightly wider towards the distal end of the leaf (Fig. 3b,c).

A GLM analysis relating the size of lesions caused by *B. cinerea* to the NO_3^- fertilization and experiment effects, along with their interaction, yielded a highly significant model (r^2=0.41, 1349 df, f=175, p<0.0001, Fig. 4a). Response curves were best fitted by a logistic function in E1 and a power function in E2 and E3. Whatever the model, the lesion size increased significantly in each experiment between 10 mM and 20 mM NO_3^- in the nutrient solution.Experimental factors generated a 2.3 fold variation in disease variability. A small but significant (p<0.0001) part of the observed lesion variability was due to a "strain" effect. When added to the GLM model, this third factor improved the model R^2 very slightly (0.44 vs 0.41). The lesions caused by strain BC1 were 34% greater on average than those caused by the other five strains tested (1875 mm^2 vs 1394 mm^2, data not shown). No significant differences were found between the five other strains (data not shown). The "strain x experiment" effect was not statistically significant (p=0.07) and individual tests for each strain all indicated significantly greater lesion sizes in experiment E1. Strain variability from *B. cinerea* was not considered further.

The same GLM model fitting performed on lesions caused by *S. sclerotiorum* yielded comparable results: there was a highly significant effect of N fertilization level on lesion size (r^2=0.58, 224 df, f=58, p<0.0001), with also highly significant effects of the individual experiments and the NO_3^- x experiment interaction. Lesion size increased with NO_3^- concentrationin the fertigation solution, by a factor of 6.8 from 950 mm^2 at 0.5 mM NO_3^- to 6500 mm^2 at 20 mM NO_3^- (Fig. 4b). Response curves were best fitted by a logistic function in experiment E1 and a Hill function in the two others. In contrast with what was observed for *B. cinerea* however, disease reached an asymptote at 10 mM and above: the disease was significantly greater at 20 mM NO_3^- in E3, but not statistically different between the two regimes in the first experiments..

Relationship between plant nutrient content and lesion development

Leaf C and NO_3^- contents (mg g^{-1} fresh weight) were not normally distributed and so were log-transformed. Amongst the plant elements measured, only the N, logC, logNO_3^-, SUC contents and the N/C ratio were consistently correlated with lesion areas (r^2>0.7) in all three experiments, for the two diseases independently (Table 3). The total sugars, FRU, GLU or hexoses (FRU + GLU) contents, as well as the P, and Mg contents were not significantly correlated with any of the two diseases, while K and Ca were correlated, but not in all experiments, with lesions caused by *B. cinerea* and *S. sclerotiorum* respectively (Table 3). Additionally, the N/C ratio and the FRU/total sugars ratio also appeared highly significant in regressions for lesion areas for the two pathogens, in all three experiments (data not shown).

As the four identified individual factors (N, logC, logNO_3^-, SUC) were correlated, we further analysed the data with type I SS assessment in repeated GLM models (24 possible combinations with 4 variables, see Materials and Methods section). For *B. cinerea*, the model with these four factors was significant (r^2=0.70, 14 df, F=5.8, p=0.01). The most significant factors were logNO_3^- (p=0.04 with type III SS) and N (p=0.08 with type III SS). Analyzing the type I SS analysis, the logC and SUC effects appeared not to be independent of the logNO_3^- effect. The N effect was independent from the logNO_3^- effect, and in regression models where both NO_3^- and N were introduced, the parameter related to N was negative.

For *S. Sclerotiorum*, the model was highly significant (r^2=0.93, 14 df, F=31, p<0.0001). The most significant factors were SUC (p=0.004 with type III SS) and N (p=0.06 with type III SS). The NO_3^- effect was not independent of the SUC effect, and the N effect was independent of the three other effects. Here again, the parameter associated to N in models was negative when the factor NO_3^- was introduced. The effect of logC was, for the two diseases, in most regressions not independent of the other effects.

Individual regressions are presented for logNO_3^-, N and SUC in Fig. 5. In all the experiments, the best model for the regression of *B. cinerea* lesions on logNO_3^- was an exponential function, with increasing disease severity at higher plant NO_3^-content (Fig. 5a). However the fitted parameters were different from one experiment to another, and little was gained by analysing the symptoms on a plant NO_3^-basis rather than on a NO_3^- concentration in solution basis. Although a negative relationship between plant SUC content and lesion area was found in each experiment (Fig. 5b), the best fits were achieved with different functions for the various experiments.. Plant N content, isolated from the other plant components, was positively correlated with *B. cinerea* symptoms (Fig. 5c).

For *S. sclerotiorum*, although the relationships between lesion size and plant NO_3^- were very strong in each experiment, the overall regression showed poor predictive capacity at high plant NO_3^-content (Fig. 5d). As stated earlier, there was a strong negative correlation between $NO_3^-$and SUC, which was closely correlated with disease intensity, either when considering individual experiments or the pooled data (Fig. 5e). The fitted functions were an exponential function for E1 and E2 experiments, and a linear function for E3. However, the MSEP of the linear and exponential models in this later experiment were very close. The relationship between disease intensity and SUC explains the relatively weak symptoms in 2010, where SUC plant contents were high, regardless of the NO_3^- fertigation regime. As for *B. cinerea*, the plant N content had poor predictive capacity on the pooled data, but total N was positively correlated with disease, when including the nitrate fraction of total N (Fig. 5f). Relating the disease to the proportion of SUC within the total sugars pool did not greatly affect the observed effect of SUC alone on *B. cinerea* or *S. Sclerotiorum* (Fig. 6a,b). However, although FRU alone was very poorly correlated with disease intensity, the ratio of FRU on total sugars fitted very well the data, following an exponential function for *S. sclerotiorum* (Fig. 6c,d). Conversely, the ratio of GLU / total sugars did not give any fit to the data (data not shown).

Discussion

Examining the effect of fertilization treatments on the plants, we observed that NO_3^- concentration in the nutrient solution limited growth up to 10 mM NO_3^-, following a typical plateau curve. Doubling the concentration from 10 to 20 mM NO_3^- did not increase growth, but led to an accumulation of NO_3^- and of reduced N. These results are in line with previous reports showing NO_3^- accumulation in lettuce leaves when its uptake rate is higher than its assimilation rate (Maynard et al. 1976). The observed consequences of N shortage or excess on plant C and dry matter accumulation relative to the plant water content have also been reported before (Seginer 2003; Burns 1994; Cardenas-Navarro et al. 1999). Less is known about the effect of Nnutrition on lettuce sugar content, but our observations seemconsistent with general considerations on the coordination of C and N metabolism (Stitt et al. 2002). In the lowest NO_3^- regime (0.5 mM NO_3^-), growth – and probably photosynthesis - were severely restricted, and the available C was stored as SUC as starch accumulation in lettuce is limited (Seginer 2003). For wheat, higher SUC content at low NO_3^- availability has already been reported (Lawlor et al. 1987). At higher regimes (2-5 mM NO_3^-), growth was still N-limited, and the availability of sugars might have reflected the availability of photosynthetic products that were not involved in proteins and organic acids metabolisms. Sugars are also known to maintain osmolarity in lettuce in the absence of NO_3^- (Blomzandstra and Lampe 1985). Significant differences were found between our experiments. Leaf NO_3^- and potassium content at 10 and 20 mM NO_3^- were higher in experiment E1, while the SUC content relative to the total sugar pool was much higher in E3 (table2). The high evaporative demand observed during E1 called for increased frequency and duration of irrigation events and, as a consequence of the constant ion concentrations in solutions, the plants were fed with relatively more NO_3^- and potassium in that experiment than in the others. Additionally, we consistently observed in the experiments a strong negative correlation between nitrate and SUC contents. These contrasting environments, in association with the nitrate nutrition treatments, allowed us to obtain a large range of C and N plant contents, which was helpful to assess the influence of primary metabolites on disease intensities.

Several differences were observed between *B. cinerea* and *S. sclerotiorum* infections. First, tissue colonization was much faster for *S. sclerotiorum*, which caused larger lesions in five days than *B. cinerea* in six days (Fig. 3).. As lesions of both fungi appeared at approximately the same time, 24-36h after inoculation, this was due to a higher mycelial expansion rate of *S. sclerotiorum*. The ability of *S. sclerotiorum* to achieve a quicker colonization of its host than *B. cinerea* had already been noticed on tomato stems (Gerlagh et al. 1996) and sunflower leaves (Dulermo et al. 2009), and mirrors the generally higher growth rate of *S. sclerotiorum in vitro*. However, although the experimental methods and environmental conditions in the growth chambers before and after inoculation were similar, the experimental effect on lesion size was not the same for the two pathogens.. Maximum lesion development for the two fungi was not found in the same experiments: lesions caused by *S. sclerotiorum* were larger in E2, while those caused by *B. cinerea* were larger – for all the tests on six strains - in "E1". The incubation conditions which conferred the plant more efficient defence against *B. cinerea* were thus not necessarily optimal against *S. sclerotiorum*, and vice-versa. Furthermore, response curves of disease severity to host NO_3^- nutrition were not identical: although both diseases were favored by high N fertilization, there was no increase of symptoms between 10 mM NO_3^- and 20 mM NO_3^- for *S. sclerotiorum*, while a significant increase in disease severity was found between 10 mM NO_3^- and 20 mM NO_3^- for *B. cinerea*.. It can therefore be concluded that symptoms of *B. cinerea* and *S. sclerotiorum* on lettuce grown at various N levels differ significantly, although higher N fertilization generally increased the severity of both disease.

Another objective of our study was to assess the effect of plant composition on disease development, regardless of fertilization treatments. Regressions of disease severity on several plant components gave a much better fit to the data than did the NO_3^- treatments. The severity of both diseases was related to the plant's content of NO_3^- , total N, SUC, and total C. The regressions on these four components explained 70% and 93% of overall disease variability in all three experiments for *B. cinerea* and *S. sclerotiorum* respectively. For *B. cinerea* the most significant factor was plant NO_3^- content, while SUC and C effects on lesion size could not be separated statistically from the NO_3^- effect. There was an influence of total N independent of that of NO_3^-, illustrated by more severe *B. cinerea* infections in experiments where total N was lower and the nitric fraction of total N higher. Lesion size was negatively

correlated with the C/N ratio, which disagree with observations showing that disease incidence after inoculation of *B. cinerea* spores on tomato leaves was positively correlated to C/N (Hoffland et al. 1999). These results as a whole show that total N, total C or their ratio, when considered alone, are not reliable predictors of plant susceptibility to *B. cinerea*. We show here that, in lettuce, NO_3^- accumulation in leaves was associated with higher disease.

In contrast with what was observed for *B. cinerea*, susceptibility of lettuce leaves to *S. sclerotiorum* was more significantly linked to sugars than NO_3^-. We observed a negative exponential relationship between leaf SUC content and lesion size, which was quite similar in the three independent experiments (Fig. 5e). Although FRU and GLU contents were negatively correlated with SUC, we did not observe any positive correlation between hexoses and susceptibility. Thus, either SUC or other metabolites whose variations mirror those of SUC are possibly linked with a decrease of lettuce susceptibility to *S. Sclerotiorum*, or a decreased aggressiveness of the pathogen. Also, ratios representing the proportion of SUC and FRU on the total sugar pool were closely linked to lesion size. It has been shown on sunflower cotyledons that *S. sclerotiorum* depletes the host from its SUC and FRU, but not its GLU (Jobic et al. 2007), during the course of infection. The involvement of sugars in plant defense has been increasingly recognized in recent years. Sugars may act either by providing C skeletons for the synthesis of defensive secondary metabolites, and/or as signals for the induction of defence-related pathways (Berger et al. 2007; Bolton 2009). In several plant-fungus interactions, SUC is known to be mobilized upon infection via an increase in acid invertase activities of both plant and fungal origins (Berger et al. 2004; Jobic et al. 2007). The use of SUC as a C source for the synthesis by the plant of defensive metabolites first requires its cleavage into FRU and GLU. If a significant involvement of SUC, via its initial transformation into hexoses, was necessary for fuelling the defence, then a lower susceptibility should have been found also at high hexose content;however neither GLU nor FRU were directly correlated to any of the two diseases. SUC is known to be a specific inductor of flavonoid biosynthesis in Arabidopsis (Solfanelli et al. 2006), and SUC-supplemented tissues activate the synthesis and accumulation of flavonoids in lupins challenged with *Fusarium oxysporum* (Morkunas et al. 2011). An enhanced production of anti-fungal metabolites in plants containing high SUC concentrations is compatible with our observations, SUC playing the role of a signaling molecule rather than that of a C source for

polyphenol synthesis. It also appeared that the respective proportions of FRU and SUC in the total sugar pool were even more closely related to the diseases than individual sugars. SUC and FRU were negatively correlated. This suggests that an induction of defense mediated by plant sugars might be controlled by the relative content in SUC and FRU, rather than their absolute concentration in the infected tissues. . Although a negative correlation between SUC content and lesion size was also found for *B. cinerea*, the relationship with SUC gave a poorer fit to the data than NO_3^- did.

The main conclusion of our work is that, at least for one of the two necrotrophs in this study, the effect of N fertilization on susceptibility appeared statistically mediated by the metabolism of sugars. As sugar accumulation is specific in each plant species, and for a given species, in its different organs, it might explain why the variation of plant susceptibility to necrotrophs in response to N fertilization is complex. Genotypes with different sugar accumulation should be useful to test a general relationship between specific sugars and resistance to necrotrophs in lettuce and other species.

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Table 1 Ionic concentrations (mmol L^{-1}) of main nutrients in the fertilization solutions (Solutions S1 to S5, with increasing NO_3^- concentration from S1 to S5) used for lettuce production.

mmol.L^{-1}	**[NO3-]**	**[K+]**	**[Ca2+]**	**[Mg2+]**	**[SO42-]**	**[H2PO4-]**
S1	0.5	11	3.25	3.5	7.25	1
S2	2	11	3.25	3.5	6.5	1
S3	5	11	3.25	3.5	5	1
S4	10	11	3.25	3.5	2.5	1
S5	20	21	3.25	3.5	2.5	1

Table 2 Leaf content (mg g^{-1} fw) of lettuce plants grown at five different NO_3^- fertilization regimes, in three independent experiments. Values are the mean of five observations. Symbols in the bottom lines indicate significant differences between NO_3^- treatments, experiments, and their interaction, in a Student Newman-Keuls test: *** $p<0.0001$; n.s.: not significant

NO_3^- concentration in solution (mM)		**N**	**NO3**	**C**	**SUC**	**GLU**	**FRU**	**P**	**K**	**Ca**	**Mg**
		(mg/g fw)	**(mg/g fw)**	**(mg/g fw)**	**(mg/g fw)**	**(mg/g fw)**	**(mg/g fw)**	**(mg/g fw)**	**(mg/g fw)**	**(mg/g fw)**	**(mg/g fw)**
0.5	*E1*	1.13	0.02	38.4	4.99	1.67	1.24	0.43	4.51	0.51	0.31
	E2	1.94	0.04	45.6	4.43	2.12	2.25	0.69	3.50	0.92	0.22
	E3	1.37	0.03	45.4	5.71	0.53	0.46	0.71	3.55	1.04	0.37
2	*E1*	1.48	0.03	33.8	4.73	4.25	4.1	0.46	4.12	0.47	0.34
	E2	2.2	0.09	29.9	4.71	4.17	4.88	0.57	2.95	0.55	0.13
	E3	1.98	0.03	42.1	6.47	1.71	1.51	0.73	3.64	0.54	0.3
5	*E1*	1.82	0.34	25.8	1.46	5.62	6.71	0.45	3.57	0.38	0.23
	E2	2.29	0.41	24.2	3.09	4.12	5.94	0.51	2.23	0.27	0.11
	E3	2.45	0.15	30.9	5.43	3.25	3.71	0.62	2.89	0.59	0.17
10	*E1*	2.14	1.51	20.8	0.68	4.07	5.07	0.43	3.43	0.28	0.28
	E2	2.46	0.71	19.6	1.34	1.95	4.31	0.52	2.08	0.22	0.13
	E3	2.68	0.61	25.9	4.45	2.31	4.28	0.53	2.49	0.21	0.08
20	*E1*	2.62	3.37	20.4	0.61	3.66	4.64	0.45	4.28	0.26	0.18
	E2	2.76	1.07	19.7	1.32	1.34	3.58	0.40	1.76	0.17	0.12
	E3	2.89	0.95	25.1	3.65	1.71	3.67	0.53	2.56	0.33	0.11
p for effects											
NO3-		***	***	***	***	***	***	***	***	***	***
experiment		***	***	***	***	***	***	***	***	ns	***
NO3- x experiment		***	***	***	***	***	***	***	***	***	***

Table 3 Coefficients of determination r^2 and probabilities of significance of linear models relating lesion size to concentrations of elements in plants (mg g^{-1} fw). Bold r^2 values indicate r^2 beyond 0.7.

		N		**NO3**		**C**		**SUC**		**GLU**		**FRU**		**P**		**K**		**Mg**		**Ca**		**Hexoses**		**Tot. Sugars**	
		r^2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*	r2	*p*
B. cinerea	E1	**0.96**	*0.004*	**0.90**	*0.014*	**0.85**	*0.026*	**0.74**	*0.063*	0.03	*0.75*	0.17	*0.49*	0.01	*0.88*	0.03	*0.76*	**0.78**	*0.048*	**0.91**	*0.011*	0.11	*0.59*	0.09	*0.63*
	E2	**0.95**	*0.005*	**0.96**	*0.004*	**0.86**	*0.024*	**0.83**	*0.031*	0.16	*0.49*	0.07	*0.65*	**0.93**	*0.008*	**0.96**	*0.003*	0.55	*0.15*	**0.86**	*0.022*	0.01	*0.92*	0.23	*0.41*
	E3	**0.76**	*0.051*	**0.83**	*0.032*	**0.77**	*0.051*	**0.83**	*0.033*	0.08	*0.62*	0.52	*0.16*	**0.72**	*0.067*	0.67	*0.09*	0.66	*0.096*	0.41	*0.24*	0.35	*0.29*	0.05	*0.71*
S. sclerotiorum	E1	**0.84**	*0.028*	**0.97**	*0.002*	**0.96**	*0.004*	**0.96**	*0.004*	0.12	*0.57*	0.33	*0.31*	0.01	*0.88*	0.25	*0.38*	**0.99**	*3E-04*	**0.96**	*0.004*	0.23	*0.41*	0.04	*0.75*
	E2	**0.73**	*0.064*	**0.80**	*0.041*	**0.78**	*0.047*	**0.92**	*0.009*	0.34	*0.3*	0.01	*0.89*	0.55	*0.15*	**0.73**	*0.064*	0.28	*0.36*	0.68	*0.089*	0.06	*0.67*	0.44	*0.22*
	E3	**0.83**	*0.031*	**0.97**	*0.002*	**0.93**	*0.009*	**0.92**	*0.009*	0.13	*0.55*	0.69	*0.08*	**0.92**	*0.01*	**0.84**	*0.019*	**0.82**	*0.032*	0.51	*0.17*	0.47	*0.19*	0.1	*0.61*

Fig. 1 Leaf area of lettuce plants grown under different NO_3^- fertilization regimes. Each curve represents an independent experiment (blue dots: E1; red squares: E2; green triangles:E3). Vertical bars represent the standard error of the mean.

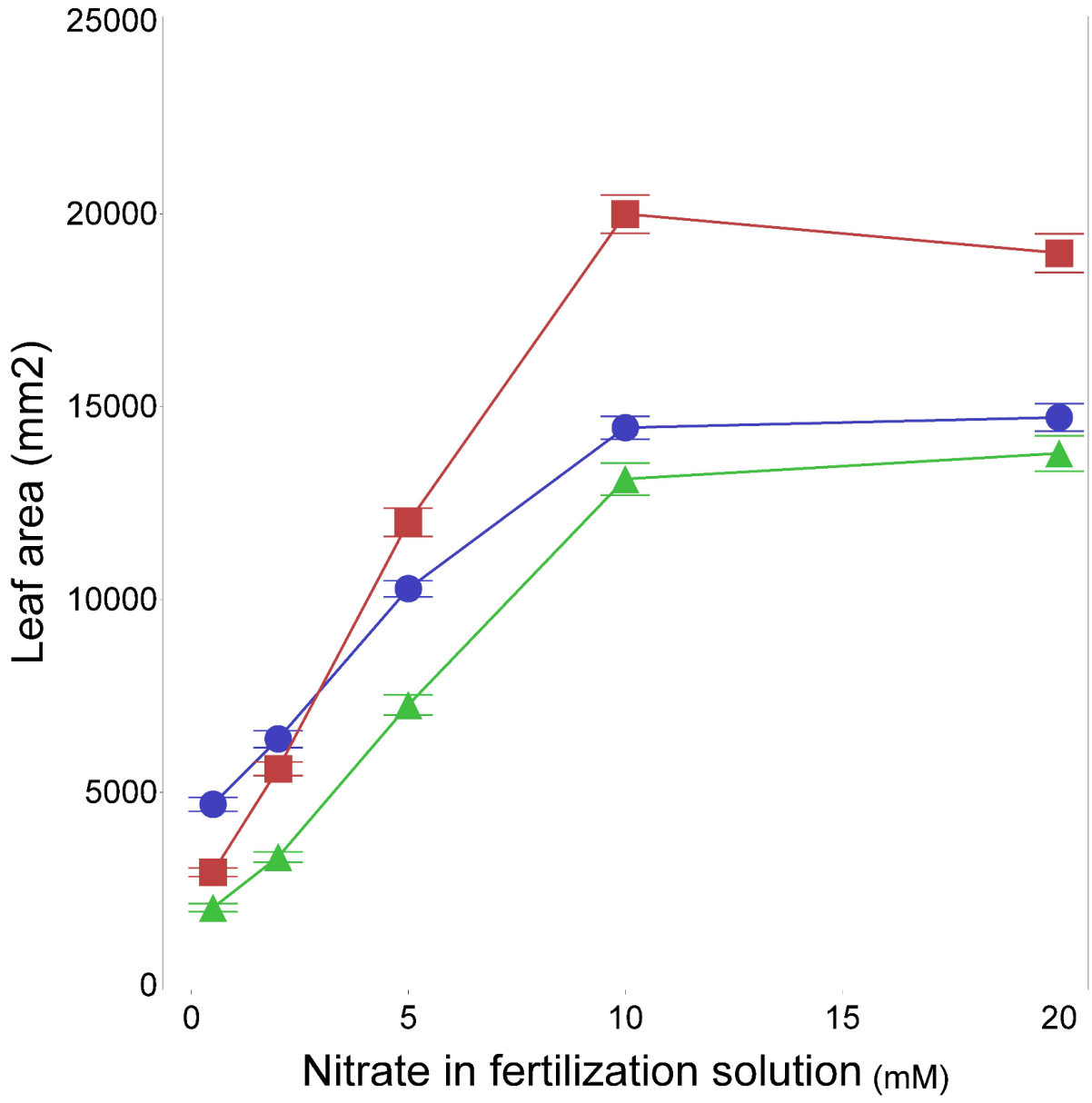


Fig. 2 Total sugars (SUC+GLU+FRU, a) and ratio of SUC/total sugars (b) in leaves of lettuce plants grown under different N regimes in three independent experiments (dots: E1; squares: E2; triangles:E3). Horizontal bars represent the mean and vertical bars the standard error of the mean.

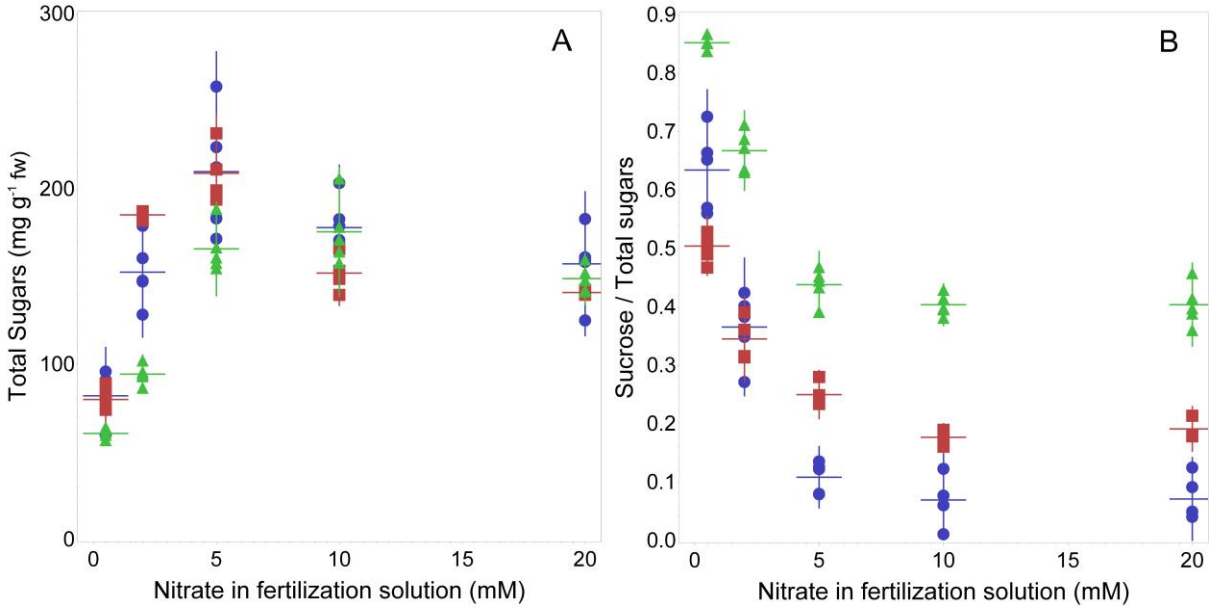


Fig. 3 Overall disease assessment with pooled data, independently of NO_3^- regimes. a: Lesion size at 6 days (*Botrytis cinerea*) or 5 days (*Sclerotinia sclerotiorum*) after infection by mycelia disks, in three independent experiments. Bars represent the mean ± standard deviation. b: Lesions caused by *Botrytis cinerea* on lettuce leaves. C: Lesion caused by *Sclerotinia sclerotiorum* on lettuce leaves.

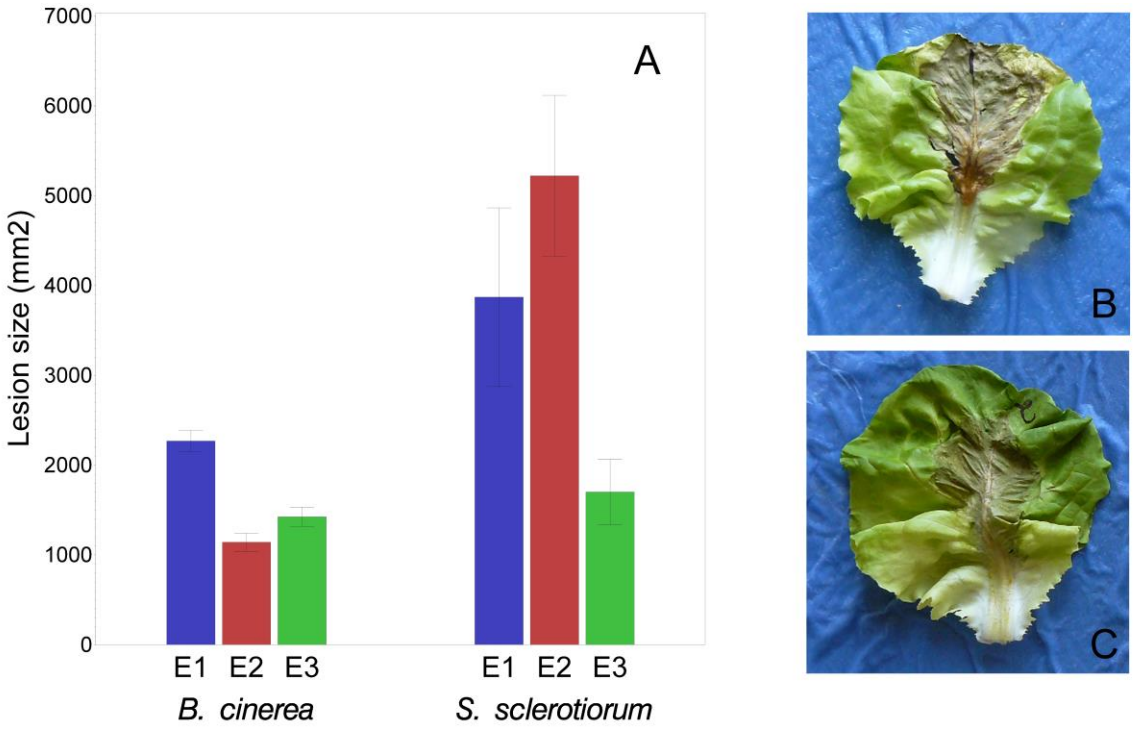


Fig. 4 Size of leaf lesions caused by *Botrytis cinerea* (a; 6 days after inoculation) and *Sclerotinia sclerotiorum* (b; 5 days after inoculation) under different NO_3^- fertilization regimes, in three independent experiments (dots: E1; squares: E2; triangles:E3). Each symbol is the mean of 90 lesion measurements for *B. cinerea* (pooled data for 6 strains) or 15 lesion measurements for *S. Sclerotiorum* (1 strain). Horizontal bars represent the standard error of the mean. Continuous lines join the symbols, dotted lines are statistical fits by non-linear functions. *Botrytis cinerea* response curves were fitted by a logistic function (E1) and a power function (E2 and E3). *Sclerotinia sclerotiorum* response curves were fitted by a logistic (E1) and a Hill function (E2 and E3).

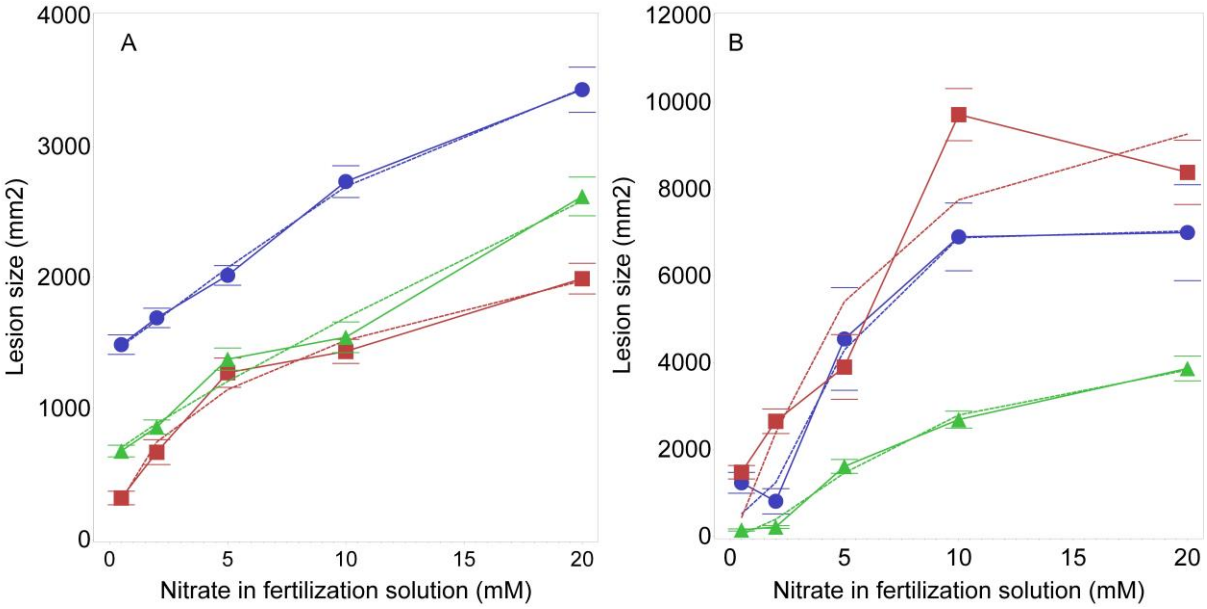
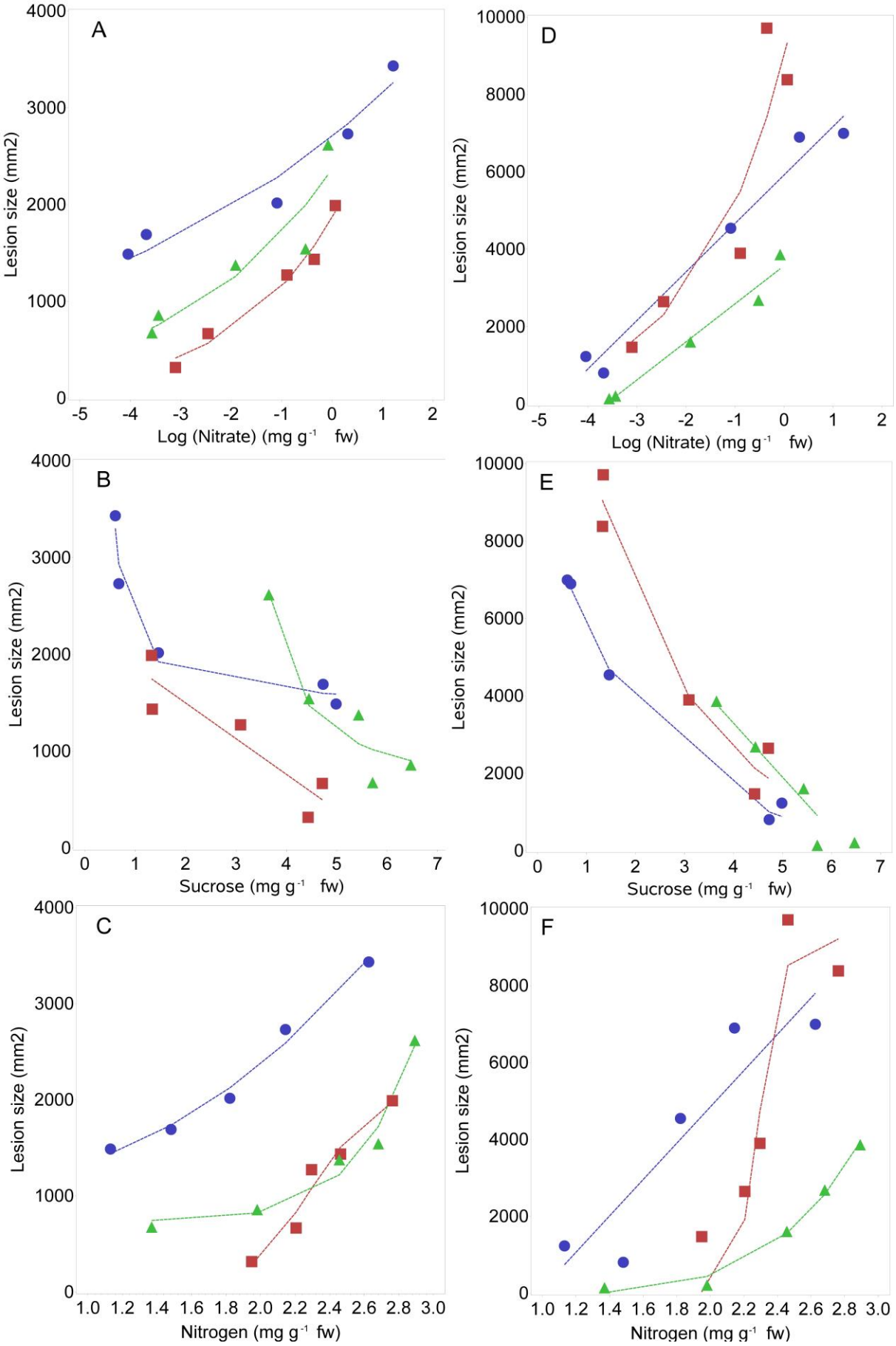
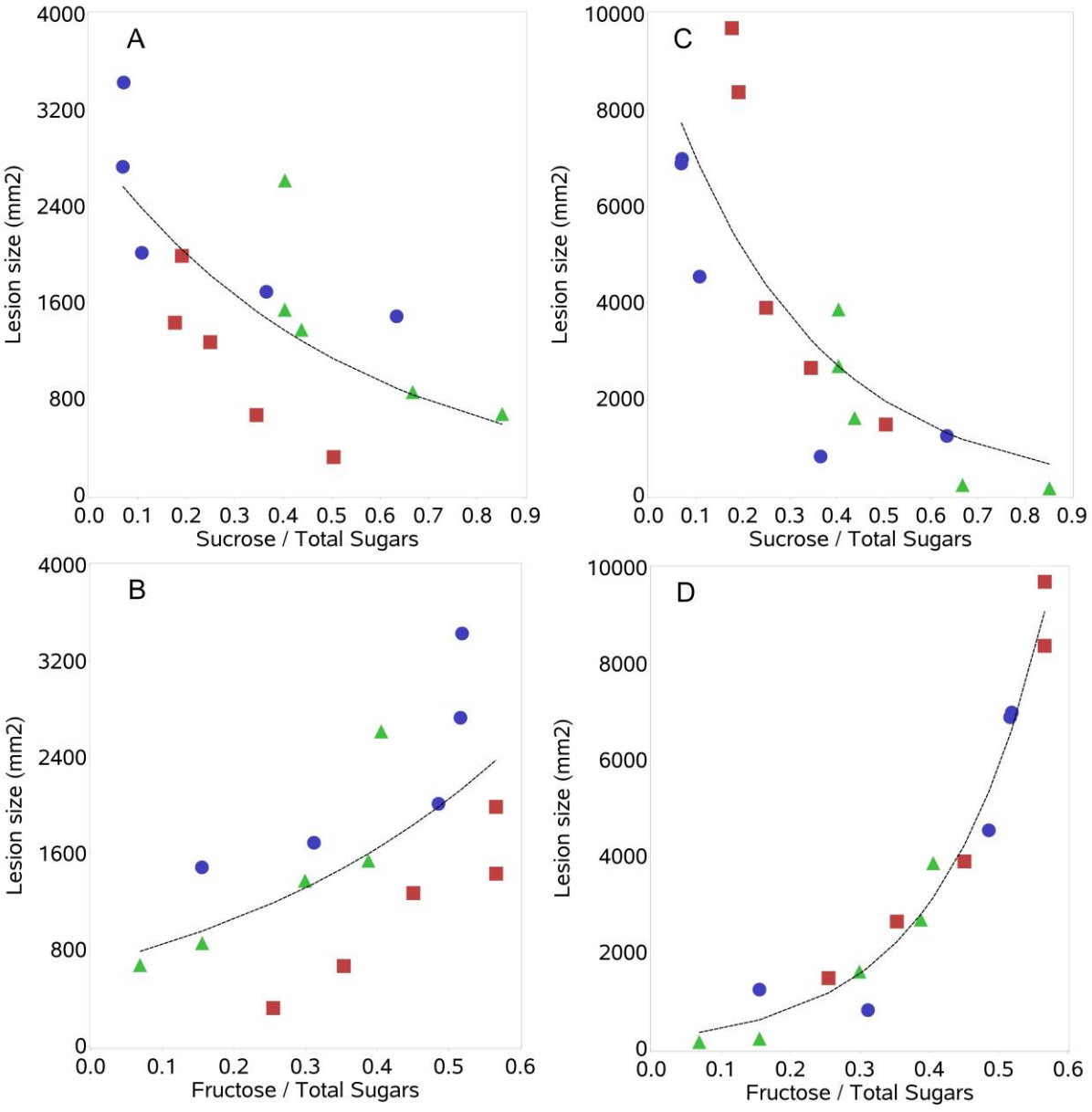


Fig. 5 Size of leaf lesions caused by *Botrytis cinerea* (a, b and c; 6 days after inoculation) and *Sclerotinia sclerotiorum* (d, e and f; 5 days after inoculation) on lettuce plants as a function of leaf contents (in $mg.g^{-1}$ fresh weight). a, d: log (NO_3^-); b, e: SUC; c, f: N. Each symbol relates the mean of 5 plant analyses to the mean of 90 lesion measurements for *B. cinerea* (pooled data for 6 strains) or 15 lesion measurements for *S. Sclerotiorum* (1 strain). Different symbols and colours represent independent experiments (dots: E1; squares: E2; triangles:E3). Continuous lines join the symbols, dotted lines are statistical adjustments by non-linear functions. Details of fitted functions are given in the text.


A
Lesion size (mm2)
Log (Nitrate) (mg g-1 fw)
B
Sucrose (mg g-1 fw)
C
Nitrogen (mg g-1 fw)
D
E
F

1

Fig. 6 Fungal lesions caused by *Botrytis cinerea* (a, b) *Sclerotinia sclerotiorum* (c,d) related to the ratio of SUC (a, c) and FRU (b, d) on Total Sugars (SUC+FRU+GLU). Each symbol relates the mean of 5 plant analyses to the mean of 90 lesion measurements for *B. cinerea* (pooled data for 6 strains) or 15 lesion measurements for *S. Sclerotiorum* (1 strain). Different symbols and colours represent independent experiments (dots: E1; squares: E2; triangles:E3). Dotted black lines are fitted exponential functions on the pooled data for the three experiments (y=k1*exp(k2*X), with X=SUC/total sugars in a and c, X=FRU/total sugars in b and d; a: k1=2926, k2=-1.88; F=55.8, p<0.0001, 15 DF; b: k1=676, k2=2.2, F=45.1, p<0.0001, 15 DF; c: k1=9659, k2=-3.2, F=44.1, p<0.0001, 15 DF; d: k1=214, k2=6.6; F=527, p<0.0001, 15 DF).



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