The impact of nitrogen form and supply rate on the growth of *Cladosporium fulvum in vitro*

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Abstract

A number of experiments were carried out to investigate the impact of different supply rate and form of nitrogen on the growth of *C. fulvum* in vitro. The results of these experiments indicated that fungal growth was affected by supply rate and form of nitrogen. Both forms of organic and inorganic nitrogen can be used by the fungus.On the other hand, the *C. fulvum* was grow better when nitrogen supplied at 5 mM in form of nitrate compared with others. Also,the highest biomass of the fungus was achieved when nitrogen had been supplied as glutamate, GABA or cas-amino acids. In conclusion, the results of this study provide an important link with the conditions that the fungus may experience during infection *in vivo*. It is clear that *C. fulvum* can utilise the major nitrogen sources available in the plant during infection and both the form of nitrogen and their supply rate are likely to affect growth of the fungus *in vivo*.

Key words: *Nitrogen, form, supply rate, Cladosporium fulvum.*

تأثير إضافة معدلات وصور مختلفة من النتروجين علي نمو فطر *Cladosporium fulvum* في المعمل

الملخص:

أجريت عدة تجارب لدراسة تأثير إضافة معدلات و صور مختلفة من عنصر النيتروجين علي نمو فطر *Cladosporium fulvum* بالمعمل. أظهرت نتائج هده التجارب إن النمو الفطري *تأثر بكلا من معدلات الإضافة* و الصورة المضاف عليها النتروجين. دلت نتائج هده الدراسات إن فطر *Cladosporium fulvum* يستطيع استخدام عنصر النتروجين في صورتيه العضوية والغير عضوية لسد احتياجاته الغذائية. من ناحية أخري كان نمو الفطر جيدا عند إضافة النيتروجين بمعدل 5 mM في صورة نثرات مقارنة مع الصور الاخري للنتروجين. كذلك أشارت نتائج هده التجارب إن اعلي معدل لنمو الفطر كان متحصلا علية عندما كان النيتروجين مضافا في صورة glutamate & GABA and caso amino acids . دلت النتائج أمكانية إيجاد رابط للتعرف علي الظروف والسلوك الغذائي الذي يسلكه الفطر أثناء الإصابة بالحقل. وأصبح من الواضح إن فطر *Cladosporium fulvum* يمكنه استخدام معظم مصادر النتروجين الموجودة والمتاحة في النبات أثناء الإصابة.

الكلمات المفتاحية: صور النيتروجين، معدل الإضافة، فطر *Cladosporium fulvum*.

Introduction:

Nitrogen is one of the most important nutrients and often limits plant and pathogen growth. It is a constituent of proteins, nucleic acids and many other important cellular compounds (Lea and Morot-Gaudry, 2001) . It represents about 1.5 - 2% of plant dry matter and approximately 16% of total plant protein (Wang *et al.*, 2001). A number of studies have investigated the impact of both nitrogen form and supply rate on plant morphology such as leaf cell number and size of sunflower (*Helianthus annuus* L.) and tobacco (*Nicotianatabacum* L.). These studies have indicated that leaf cell number and size of sunflower were significantly increased as a result of feeding plants with a high supply rate of nitrogen compared with plants treated with a low supply rate of nitrogen (Trapani et al., 1999).

Also it has been reported that abundant nitrogen supply increases the number of meristems produced by plants and their growth, thus encouraging shoot formation and growth in most plants (Lawlor et al., 1988; Lawlor et al., 1989) The impact of nitrogen form and supply rate on the plant growth has received great attention by plant physiologists, biologists and ecologists; and has increased the current understanding of nitrogen metabolism in plants. On the other hand,all sources of nitrogen for fungal growth are derived from the plant. Such sources could include ammonia, nitrate, amino acids and other small molecules and proteins (Solomon *et al*., 2003). Although fungal pathogens are able to use a wide range of nitrogen forms to support their growth there is strong evidence to demonstrate that the susceptibility of plants to pathogens is affected by nitrogen form and supply rate (Huber and Watson, 1974; Hoffland *et al.*, 2000a; Snoeijers *et al.*, 2000; Dadd, 2002).The utilization of nitrogenous compounds by pathogen will led to a clear understanding of infection development in plant and the way of control it.

Materials and methods:

***In vitro* culture of *Cladosporium fulvum*.**

Potato Dextrose Agar (PDA) plates were prepared by mixing 6g of Potato Dextrose Broth (PDB) (Sigma-Aldrich, Steinheim, Germany) and 15g of bacto agar in 1 litre of distilled water and then autoclaved at 121ºC for 15 min. Plates were poured and allowed to solidify in a HeraeusLaminr Air Saftey Cabinet HB2436. Plates were then inoculated with 100µl of concentrated spore suspension of *C. fulvum*. Plates were sealed with parafilm (American National Can, USA). All plates were transferred to an incubator at 24˚C for 18 days to allow sporulation to occur.

The effect different supply rate and forms of nitrogen on the growth of *C.fulvum*in vitro.

An initial experiment was carried out to determine the effect of three forms of nitrogen, (nitrate, ammonium and ammonium nitrate) at different concentrations (0.1, 0.2, 0.5,1,2,5 and 10 mM) on the growth of the fungus on solid media. The media consisted of modified B5 medium (Gamborg*et al.*, 1968) containing macronutrients (0.96 mM $NaH_2PO_4.2H_2O$, 0.09 mM Na-EDTA, 0.76 mM K_2SO_4, 0.10 mM $FeSO_4$ and 0.006 mM KI) micronutrients ((44.8 mM$MnSO_4.4H_2O$, 6.9 mM $ZnSO_4.7H_2O$, 48.52 mM H_3BO_3 , 1.03 mM $Na_2MoO_4.2H_2O$, 0.1 mM Cu $SO_4.5H_2O$, 0.17 mM $CoCl_2.2H_2O$) and agar (pH 6.5). The solution was autoclaved for 30 minutes and allowed to cool in a water bath at

60 ^{o}C. Twenty five ml of filtered sterilized 1M sucrose was added as a carbon source giving a final concentration of 50 mM sucrose. Three forms of used. Each form was added to B5 agar to give a final concentration of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 or 10 mM nitrogen. A plate without nitrogen was also established as a control. The different nutrient media were added to 24 well cell culture plates (Nunc.Inc, Roskild, Denmark).Plates were left to solidify before inoculating them with 50µl of a *C. fulvum* spore suspension (10^{6}spore ml^{-1}) prepared as described in section.2.1. Plates were sealed with parafilmand then transferred to an incubator at 23°C for 12 days. Photographs of the extent of fungal growth were taken 12 days post inoculation using a CCD camera (UVP Laboratory Products, EpiChemi II Darkroom).

The impact of nitrogen forms on the growth of *C. fulvum* in liquid culture.

The aim of this experiment was to investigate the growth of the fungus on different forms of nitrogen including nitrate, ammonium, ammonium nitrate, glutamate, γ amino butyric acid (GABA), succinate, glycine and cas-amino acids. In addition, two control treatments, water and potato dextrose broth (PDB), were used. Modified B5 media (without agar) was prepared as described in section 2.2. Filter sterilized glucose was added as the carbon source to give a final concentration of 50 mM. Each nitrogen source was added to the modified B5/glucose media to give a final concentration of 5 mM. The pH of the media was adjusted to 6.5 for all nitrogen sources.One hundred microlitres of media containing a specific source of nitrogen was transferred into wells of a 96 well cell culture plate (Corning. Inc, New York, United States).

Spores of *C. fulvum* were prepared as described in section 2.1. Spore suspension was centrifuged at 1380 ×g for 5 minutes. The spore suspension was resuspended in either sterilized distilled water (SDH_2O) or inoculating fluid (IF) (Biolog, Hayward, U.S.A). An aliquot of spore suspension was added into each well of the 96 well plates contained to give a final concentration of 2.4× 10^{4} spore ml^{-1}. Plates were sealed with parafilm paper, and then transferred to incubator at 23°C for eight days. The absorbance of the plates was measured 1, 2, 3, 4, 5, 6, 7 and 8 day post inoculation at wavelengths of 520 and 650 nm using a plate reader (Anthos HT III, Labtec, Salzburg, Austria).

The impact of different nitrogen sources on the biomass of the fungus *C. fulvum in vitro.*

The aim of these experiments was to determine fungal biomass when grown in modified B5 media containing sucrose as a carbon source and different sources of nitrogen (nitrate, ammonium, glutamate, GABA, glycine and Cas-amino acids). Modified B5 media (without agar) was prepared as described in section 2.2. B5 media was transferred into a 250 ml conical flask and autoclaved. Filter sterilized sucrose was added as the carbon source to give a final concentration of 25 mM. Each nitrogen source was added to the modified B5 media to give a final concentration of 5 mM. the final volume in each flask was 50 ml and the pH of the media was 6.5 for all nitrogen sources.One ml of spore suspension containing (10^{6}spore ml^{-1}) was added to each flask. Flasks were placed on shaker (130 rpm) in the light and incubated at 23°C for 6 days. Four replicate flasks were established for each treatment.After 6 days the fungal mycelium was harvested by filtering through a funnel contained pre-dried and weighed filter paper.

Each filter paper was dried in an oven at 55°C for 48 hours before weighing them. The dry weight of fungus was calculated by subtracting the initial weight of filter paper from the final weight after drying.

Results:

The impact of nitrogen form and supply rate on the growth of *C. fulvum in vitro* (agar plates).

To assess the effect of nitrogen form and supply rate on mycelium development, spores were grown for 12 days on modified Gamborg's B5 medium containing 0 - 10 mM nitrogen supplied as NO_3^-, NH_4^+ or in combination as NH_4NO_3 (Figure1).Growth was strongly affected by nitrogen supply rate. Mycelial development was limited at 0.1 mM, and 0.2 mM nitrogen irrespective of the form of nitrogen and the mycelium had a purple appearance (Fig 1). At concentration of 0.5 mM nitrogen and above mycelium developed and covered the surface of the growth well but growth was best at 5 mM and 10 mM nitrogen. There was relatively little impact of alerting nitrogen form except that at the higher concentrations of nitrogen (5mM and 10 mM) that the fungus slightly better when supplied with nitrate or ammonium nitrate rather than with ammonium alone. This experiment showed that 5mM nitrogen was optimal for fungal growth**.**

The impact of nitrogen form on the growth of *C. fulvum in vitro* (liquid culture).

The growth of fungus when supplied with different forms of nitrogen was assessed by measuring the increase in absorbance at520nm or 650nm for 8 days post inoculation. Figure 2shows examples of two 96 well plates where the spore suspension was made using dH_2O (A) or inoculating fluid (IF) supplied with the Biology plates (B). There was no difference in the appearance of the fungal mycelium when either dH2O or IF was used.

Figure2 illustrates the effect of the different forms of nitrogen on the colour of the fungal mycelium which ranged from opaque to brown/yellow when grown on PDB. In order to determine whether different colour would interfere with the measurement of biomass by absorbing light differentially, the absorbance of the plates was measured at two different different wavelengths, 520 nm and 650 nm. The ratio of the absorbancies at these two wavelengths is shown infigure 3. This figure shows that that the ratio of the absorbancies was for a given substrate across the time period and suggests that colour was not inteferring with the absorbance measurements. Measurements were therefore made at 520 nm.

Figure4 shows the growth of the fungus on different forms of nitrogen using either water (A) or IF (B) to make the spore suspension. The inoculating liquid had no effect on the subsequent growth of the fungus. As the experiment was carried out over 8 days growth had not reached a plateaux and therefore sigmoidal curves were not fitted to the data. The highest growth rate of the fungus was detected on cas-amino acids, glutamate, GABA, glycine or PDB (figure 4). The fungus also grew when supplied with inorganic nitrogen (nitrate, ammonium and ammonium nitrate) but less rapidly. The results of this experiment have clearly indicated that the fungus utilized organic nitrogen more effectively than inorganic nitrogen (figure 4).

The impact of different nitrogen sources on the dry weight of *C. fulvum*.

Figure 5 shows the biomass (dry weight mg) of the fungus grown on the different nitrogen sources. The highest biomass was achieved when nitrogen was supplied as glutamate, GABA or cas-amino acids. The fungus also grew well when supplied with glycine or nitrate (fig. 5). The poorest growth occurred when ammonium was used as the nitrogen source. The biomass was only 15% of that achieved with glutamate (fig. 5).

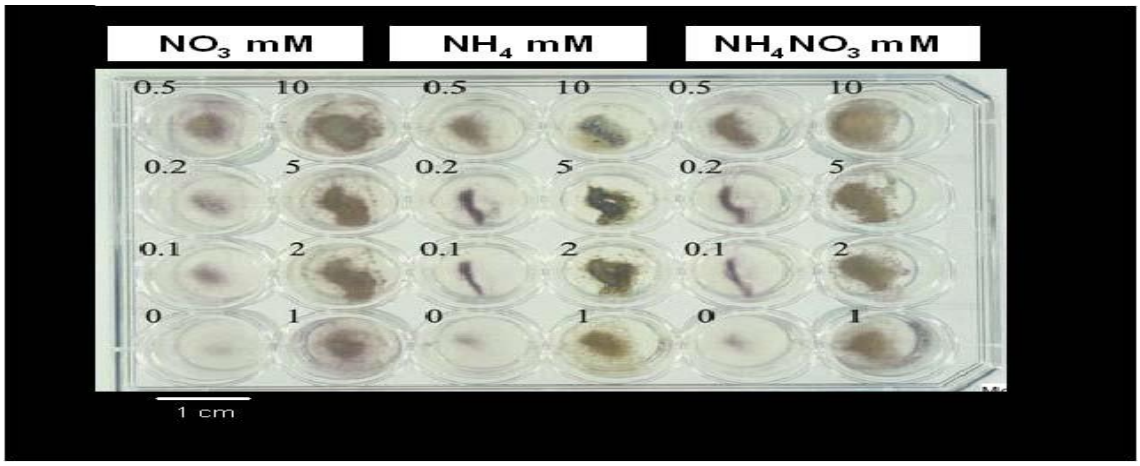

Fig. 1: The visible growth of *C. fulvum* on modified B5 media containing different forms and supply rates of nitrogen in 12 days post inoculation. the different numbers represent the concentration of nitrogen (mM). The fungus was supplied with 50 mM sucrose as a carbon source.

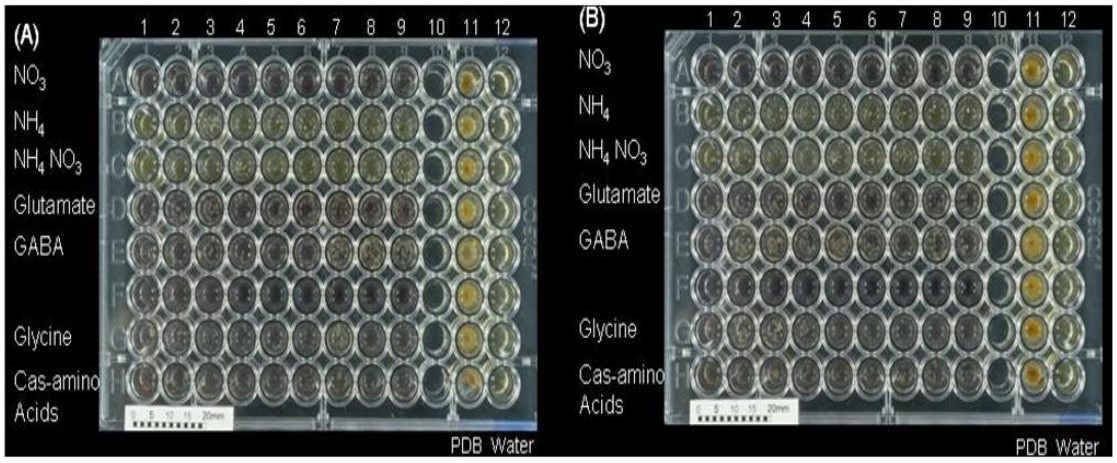

Fig. 2: The appearance of *C. fulvum* when grown in the presence of different nitrogen sources in liquid culture in 96 well plates for eight days. The concentration of each nitrogen source was 5 mM and carbon was supplied as glucose at a concentration of 50 mM.

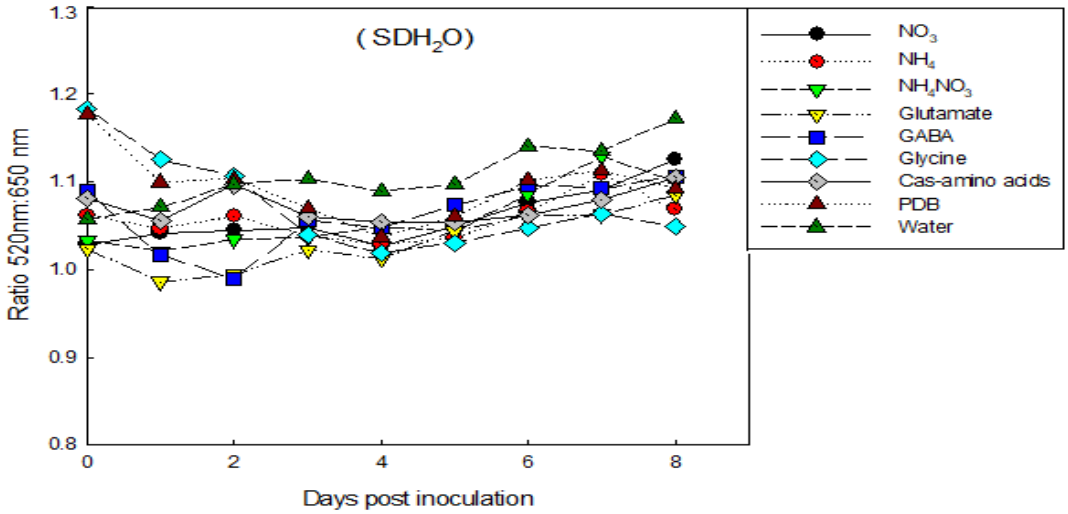

Fig. 3: The 520nm to 650nm absorbance ratio of *C. fulvum* mycelium when grown in the presence of different nitrogen sources in liquid culture in 96 well plates for eight days. The concentration of each nitrogen source was 5 mM and carbon was supplied as glucose at a concentration of 50 mM.

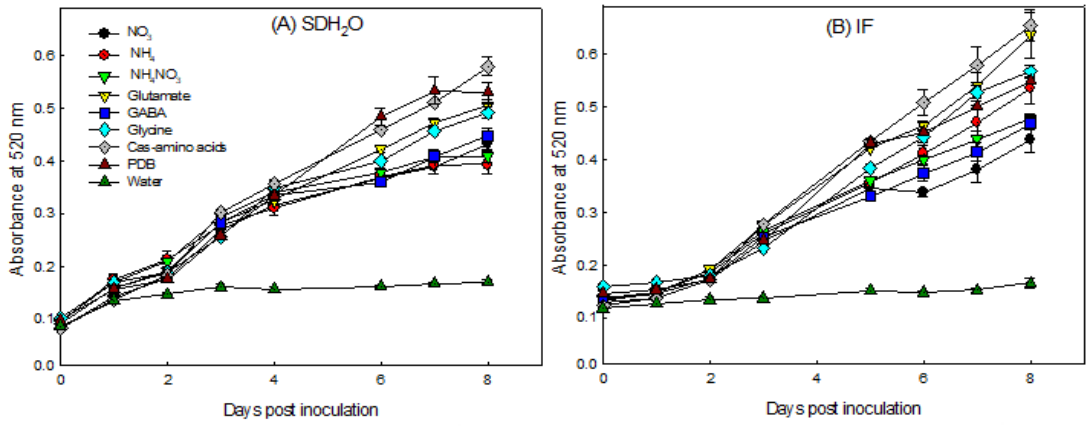

Fig. 4: The growth of *C. fulvum*on different sources of nitrogen eight days post inoculation (DPI). The concentration of each nitrogen source was 5 mM and carbon was supplied as glucose at a concentration of 50 mM.Each data point is the mean of three replicates ± S.E.

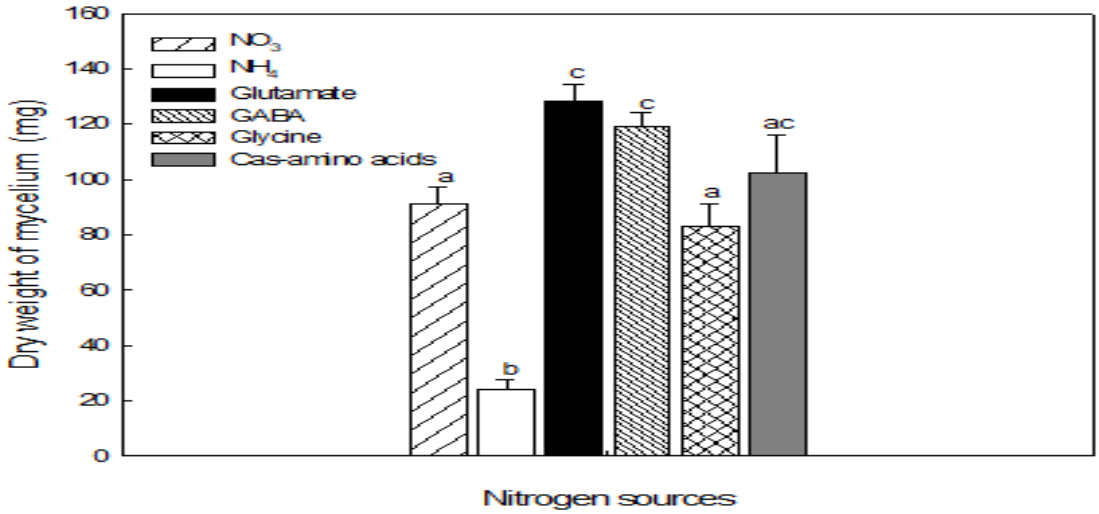

Fig. 5: The biomass (mg dry weight) of *C. fulvum* after six days growth in modified B5 media containing different nitrogen sources. Values are means ± standard error of four independent flasks. Bars marked with the same letter do not differ significantly (Tukey's multiple comparison test, df= 5, f=22.18 and p<0.001).

Discussion:

To what extent does nitrogen form and supply rate affect the growth of *C. fulvum in vitro*?

In order to investigate the impact of nitrogen form and supply rate on the growth of *C. fulvum*,experiments were performed in which the fungus *C. fulvum* was grown on modified B5 media containing different forms and amounts of nitrogen. The fungus grew best when supplied with 5 - 10 mM nitrogen and it grew most rapidly when supplied with complex and/ or organic forms of nitrogen e.g. cas-amino acids, potato dextrose broth (PDB), glutamate, GABA and glycine. The fungus was also able to utilize, but to a lesser extent, inorganic nitrogen e.g. nitrate, and ammonium nitrate. It grew least well on ammonium possibly due to a shift in pH. A similar pattern was observed when fungus biomass was harvested after 6 days growth in liquid culture with the different sources of nitrogen.These results have confirmed that the fungus can utilise both organic and inorganic nitrogen sources which are likely to be found in tomato apoplast and are consistent with the study of (Solomon and Oliver, 2001).

Nitrogen metabolism and its regulation has been studied extensively in a number of fungi particularly *Aspergillusnidulans* and *Neurosporacrassa*. Certain nitrogen compounds, ammonia, glutamine, and glutamate are preferentially used by these fungi, but when these primary nitrogen sources are not available or in too low a concentration to meet the growth requirements of the fungi, a number of different nitrogen sources can be used, e.g., nitrate, nitrite, amides, most amino acids and proteins (Marzluf, 1997; Christensen *et al.*, 1998; Tao and Marzluf, 1999; Wipf *et al.*, 2002). This is also likely to be true for *C. fulvum*.

In order, to get better understanding of the nitrogen requirement of *C.fulvum* and the availability of nitrogenous compounds during infection *in vivo*, Oliver and co-workers (2001) used an apoplast-infiltrating technique to obtain apoplastic fluid from infected plants. This was anlaysed and revealed that the concentrations of most amino acids and total nitrogen content of the tomato leaf apoplast increased during infection in a compatible interaction (Solomon and Oliver, 2001). In addition, to the organic nitrogen compounds found in tomato leaf apoplastic fluid, inorganic nitrogen e.g., nitrate was detected at a contentration of approximately 4.5 mM. The increase in the concentrations of most amino acids (except cysteine and tryptophan) began 7 days post inoculation and reached concentrations 0.1 to 0.7 mM. The highest concentration of any nitrogenous compound was γ-amino butyric acid (GABA) at around 2.5 mM(Solomon and Oliver, 2001). A further investigation has shown that GABA is a major nitrogen source in the tomato apoplast during infection by C. fulvum(Solomon and Oliver, 2002).

The utilization of organic nitrogen particularly the non-protein amino acid GABA by *C. fulvum* has been reported in this study. Although, GABA is typically found in low levels in plant tissues, the concentration of GABA can increase several folds in response to many stimuli, including heat shock, cold stimulation, phytohormones, pathogen infection and mechanical stimulation such as insect attack and wounding (Shelp *et al.*, 1999a; Kinnersley and Turano, 2000). In addition, it has recently been shown in tomato fruits under conditions of carbohydrate depletion of can also induce its formation (Baldet *et al.*, 2002). The metabolism and functions of GABA have been studied by a number of groups including (Kinnersley and Turano, 2000; Solomon and Oliver, 2002; Bouche and Fromm, 2004; Fait *et al.*, 2005)and have indicated that GABA, which is the product of glutamate decarboxylation, is metabolised via a short pathway composed of three enzymes including glutamate decarboxylase (GAD), GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) (Shelp *et al.*, 1999b; Bouche and Fromm, 2004). In addition, recent evidence suggest that GABA may also act as a long distance signal between plant organs resulting in the up-regulation of genes involved in nitrate influx in plant roots (Beuve*et al.*, 2004). Such a role for GABA may be very important in plants infected with biotrophic fungi, both for the maintenance of plant nitrogen metabolism and ultimately for the fungus which is acting as a sink for nitrogen compounds. It would be interesting to determine the concentration of GABA (and other nitrogen compounds) in phloem exudates and in apoplastic fluid of plants grown with different forms and supply rates of nitrogen in the presence and absence of *C. fulvum* to determine whether GABA (and other nitrogenous compounds) plays a major role in regulating nitrogen metabolism in diseased leaves as well as serving as a source of nitrogen for fungal nutrition.

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