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Effect of nitrogen fertilization on cucumber downy mildew

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Abstract

The objective of this study was to examine the effect of different concentrations of nitrogen on downy mildew severity on cucumber plants. Nitrogen (100-600 mg L^{-1}) was applied to plants grown in pots under greenhouse conditions through irrigation water. The cucumber leaves were inoculated with a zoospore suspension of *Pseudoperonospora cubensis*, the causal agent of powdery mildew. Disease symptoms in plants treated with 300 mg L^{-1} nitrogen were significantly lower and 24% severity reduction was recorded. A positive effect on the leaf area was also noticed in cucumber plants with the application of 300 mg L^{-1} nitrogen. The results indicated that a cubic regression curve can be fitted to the disease progress for downy mildew, regardless of N treatments. Furthermore, the N enrichment affected the NH_4-N and NO_3-N content in leaves and soil as well as P content in the leaves. The application of 100 mg L^{-1} N significantly reduced Mn content in leaves. Therefore, nitrogen supply in the form of ammonium nitrate fertilizer could be considered as an efficient method for the control of downy mildew of cucumber.

Key words: Disease control; inoculation; lesion area; nutrients; *Pseudoperonospora cubensis, Cucumis sativus.*

Introduction

Downy mildew is one of the most destructive diseases of cucumber (*Cucumis sativus* L.), causing major yield reductions (Neykov and Dobrev, 1988; Lindenthal *et al.*, 2005). The disease is caused by the obligate biotrophic oomycete *Pseudoperonospora cubensis* (Berk. and Curt.) Rostovzev. Because of the difficulty in its control many studies have examined fungicides to control downy mildew in Curcubitaceae (Keinath *et al.*, 2007; Bagi *et al.*, 2014) while alternative means for disease control are being investigated (Calcante *et al.*, 2012).

A number of factors can influence the occurrence and severity of downy mildew in cucumber plants such as the environmental conditions (temperature and humidity), crop establishment and management practices (Zitter *et al.*, 1996). Among these factors one could be the inorganic fertilizer supply. It is well known that nutrients and the incidence of plant diseases may have complex interactions. In particular, a specific nutrient can decrease the severity of a disease but can also increase the severity of other diseases or have a completely opposite influence in a different environment. Therefore, the correct management of nutrients may be useful for the development of control strategies in sustainable agriculture (Reuveni and Reuveni, 1998; Walters and Bingham, 2007; Dordas, 2008; Aqueel and Leather, 2011; Sharma *et al.*, 2012).

According to Mandal *et al.* (2008), the highest level of nitrogen fertilization increased the incidence of downy mildew on isabgol (*Plantago ovate* L.) and the same conclusion was reported for apple scab (Leser and Treutter, 2005) and tomato powdery mildew (Hoffland, 2000). In contrast, Sivaprakasam (1974) stated that downy mildew of pearl millet (*Pennisetum glaucum* L.) was not affected significantly by nitrogen nutrition. However, Bains and Jhooty (1978) have reported that downy mildew symptom severity caused by *P. cubensis* in muskmelon (*Cucumis melo* L.) was lower on plants grown in systems with a high nitrogen input. Besides, Rotenberg *et al.* (2005) reported that total soil N content was inversely related to cucumber angular leaf spot incidence. Kolota and Osinska (2001) observed that downy mildew was significantly decreased on cucumber leaves by applying a foliar fertilizer containing 11 essential nutrients for plant growth.

The host-pathogen associations are rather complex and as Hoffland (2000) reported, the balance between nutrition and tolerance-related factors of tomato depend on the invading pathogen. In addition, soil and leaf nitrogen status was associated with plant disease (Walters and Bingham, 2007; Dordas, 2008). Aphid population management (Aqueel and Leather, 2011) may also interact with downy mildew control. The form of nitrogen and the ratio of NH_4-N to NO_3-N play an important role in plant disease as Zhou and coworkers (2017) reported recently but since none of these forms controls all diseases in any group of plants each disease must be studied individually. Although the above data showed that a great deal of information is available on various pathosystems, the cucumber-*P. cubensis* has not been extensively studied. There are thus complex relationships between type and time of fertilizer application and various cucumber diseases (Reuveni and Reuveni, 1998). The objectives of this study were to (a) examine the effect of six nitrogen concentrations in the fertigation water (100 to 600 mg L^{-1} N) on downy mildew development on cucumber plants and (b) investigate the impact of nitrogen treatments on leaf nutrient concentration and whether this is related to the rate of disease progression.

Materials and methods

Experimental design: Cucumber (*Cucumis sativus* L. cv. Knossos) plants (short cucumbers, length 15-22 cm, diameter 3.5-5.5 cm) were grown under natural light during spring period in an unheated plastic greenhouse in Crete, Greece. Average minimum and maximum air temperatures during this period were 14 and 32 ºC, respectively. Plants were grown in plastic pots (7 L volume, 23.5 cm diameter x 22 cm depth) containing a surface layer of soil [sandy loam, pH=7.4, electric conductivity (EC) =2.6 mS/cm, total calcium carbonate ($CaCO_3$) 24%] sieved at 4 mm particle size.

Six nitrogen concentrations (treatments) were tested in a randomized block design with four replicated plots. Each plot consisted of six pots (derived as four replications/treatment x six pots/replication for each N treatment) which were kept 50 cm apart. The distance between the plots was 80 cm and the replicates were 100 cm from one another. Thus, the experimental treatments consisted of six total N concentrations, 100, 200, 300, 400, 500 and 600 mg L^{-1} N (with constant NO_3-N: NH_4-N ratio 6:1) applied to plants just after transplantation (2 weeks after sowing), through fertigation. These rates were chosen as the minimum dose rates and the extremely maximum of the ordinary nitrogen fertilization rates of cucumbers plants. In order to maintain the required nutrients concentration in the soil solution in each pot, the quantity of solution added (500 mL) was determined by the point where run off from the pots occurred. The rate of nutritional additions was increased according to the plants' water requirements (from three times on a weekly basis at the beginning to twice per day at the end of the experiment). Under the fertigation scheme, P and K were added to the above solutions in the same standard concentration (50 and 200 mg L^{-1}, respectively), so the plants received a balanced nutrition. Ammonium nitrate (33.5-0-0), nitric acid (HNO_3), orthophosphoric acid (H_3PO_4) and potassium nitrate (13.5-0-46.2) fertilizers were used in relevant proportions to achieve the desirable concentrations (Table 1).

Table 1. Composition of the nutrient solution applied to cucumber plants (cv. Knossos) with increasing N concentrations at a constant NO_3:NH_4 ratio of 6:1

N		NO_3	NH_4	NO_3NH_4	HNO_3	KNO_3	H_3PO_4
ppm	mM	mmol L^{-1}					
100	7.1	6.1	1.0	1.0	0	5.1	1.61
200	14.3	12.2	2.0	2.0	5.1	5.1	1.61
300	21.4	18.4	3.1	3.1	10.2	5.1	1.61
400	28.6	24.5	4.1	4.1	15.3	5.1	1.61
500	35.7	30.6	5.1	5.1	20.4	5.1	1.61
600	42.9	36.7	6.1	6.1	25.5	5.1	1.61

Plants were grown in a vertical scheme (modified 'umbrella' system) up to the horizontal wire (approximately 2.1 m height) while lateral shoots were pruned up to the first 0.5 m, and standard cultivation practices were used without any insecticide/fungicide application.

Artificial inoculation: All the plants were inoculated with *P. cubensis*, three weeks after nutrient treatments started. The inoculum (which was obtained from freshly sporulated lesions on leaves, two weeks after their inoculation) was adjusted to a concentration of 15×10^4 zoospores/mL with a hemocytometer. Five droplets (10 µL each) of the above suspension were applied on the upper surface of the fully expanded leaf(fifth leaf from the top of each plant) properly marked in order to be distinguishable during the inoculation. After inoculation, the plants were kept in a humidity chamber (average RH=95% and temperature at 25 ºC) for 24 h in the dark to facilitateacclimatization. These conditions were necessary for disease development because optimum sporulation occurs when the temperature is about 20 ºC and RH > 95% (Zitter *et al.*, 1996; Thakur and Mathur, 2002). Then, the plants were transferred to the greenhouse in their final positions and a week later, the first symptoms in form of lesions were observed.

Disease assessment: Digital images of all the infected leaves [n=4 independent leaves (in different plants) per treatment] were taken 10 days after inoculation, considered as the initial infection. Five series of images (samplings) were made: on the 2nd, 3rd, 5th, 7th, and 11th day after the first sampling. The total leaf area as well as the lesion area was measured in each sampling using special software (Bersoft Image Measurement, 4x Professional) and the results were reported as measured area (in cm^2). The incidence of downy mildew was also determined from the ratio of infected leaf area to the total leaf size of cucumber plants and expressed in percentage. The area under disease progress curve (AUDPC) was estimated to quantify disease progression over time according to Madden *et al.* (2007).

Determination of nutrient elements in plant and soil samples: In order to determine macro- and micro-elemental status affecting leaf and lesion development, leaf and soil samples (n=4, whereas each replication consisted of a bulk of 4-6 leaves/soil from different plants/pots respectively) were taken two days before the plant inoculation. Wet oxidation was used to determine the nutrients in the leaf samples. The NH_4-N and NO_3-N of leaves was extracted with KCl. The ammonium acetate extraction method was used for the soil potassium. The determination of NH_4-N and NO_3-N was made by using the direct distillation method. Calcium, magnesium, iron, manganese, copper, and zinc were determined with atomic absorption spectrophotometer (Perkin Elmer 2100, USA), phosphorus with a spectrophotometer (Pye Unicam Hitachi U-2000, Tokyo, Japan) by using the phosphomolybdovanadate method. Potassium was determined with the flame photometer (JENWAY, PEP-7 Jenway, Dunmow, UK).

Statistical analyses: The whole experiment was carried out twice. Slightly different assessments were done in the first and second experiment and we were able to get better temporal (time) data in the second one than the first one. So, the second experiment was further analyzed and presented here. Analysis of variance was used to detect differences in disease incidence as well as leaf area and soil nutritional status between treatments [six nitrogen concentrations x four replicates (plots) with six plants-pots per replication]. Treatment means were compared by Duncan Multiple Range Test (DMRT). Linear regression analysis was used to model the percentage of disease until the 7th day of the experiment. Cubic regression analysis was used to model the leaf and lesion area for the entire experimental periods (including the 11th day).

Table 2. Mean leaf area (cm^2) produced in greenhouse cucumber plants (cv. Knossos) grown in different nitrogen doses (100-600 mg L^{-1} N) in soil, at 12, 13, 15, 17 and 21 days post leaf inoculation *Pseudoperonospora cubensis*

N (mg L^{-1})	leaf area (cm^2) on different days				
	1	2	3	7	11
100	157.64 c[a]	174.31 c	190.54 d	219.04 d	220.78 d
200	212.15 b	226.97 b	249.54 c	259.67 c	279.04 c
300	218.33 b	241.22 ab	270.35 b	296.01 b	303.64 b
400	215.02 b	238.27 b	269.21 b	292.70 b	302.10 b
500	221.90 b	233.86 b	265.38 bc	284.44 b	293.95 bc
600	239.63 a	261.47 a	289.31 a	314.00 a	324.07 a

[a] Means (n=4) in columns followed by the same letter are not significantly different, (at $P \leq 0.05$) according to Duncan's multiple range test.

Results

Effect of nitrogen fertilization on leaf area and downy mildew progress: The application of different N doses to the cucumber cultivation affected the leaf area significantly. Thus, the leaf area increased with increasing nitrogen levels in the soil (Table 2). The application of 600 mg L^{-1} N resulted in the biggest leaves which were significantly different than other treatments. The nitrogen concentrations between 300-500 mg L^{-1} produced leaves of similar surface areas (Table 2).

The respective lesion area caused by *P. cubensis* was the lowest in the 300 mg L^{-1} N application up to 11 days post-inoculation, when compared with other treatments (Figure 1). Indeed, with low N levels (100-300 mg L^{-1}), the diseased area development was reduced mainly at the initiation (up to 2 days) of infection and subsequently at the end of the experiment (at 11 days). The linear model (Figure 2) interprets the percentage of disease progress until the 7th day (see R^2 in Table 3). According to slope and SE, there was a clear difference between 100 mg L^{-1} N (high growth rate) and 300 mg L^{-1} N (low growth rate). This was confirmed on the final sampling date (Table 4), where the final infection percentage was the lowest (74%) in the 300 mgL^{-1} N and the highest (97%) in the 100 mgL^{-1} N. The maximum severity reduction (24%) was noticed with the application of 300 mg L^{-1} N and AUDPC values differed significantly among treatments (Table 4).

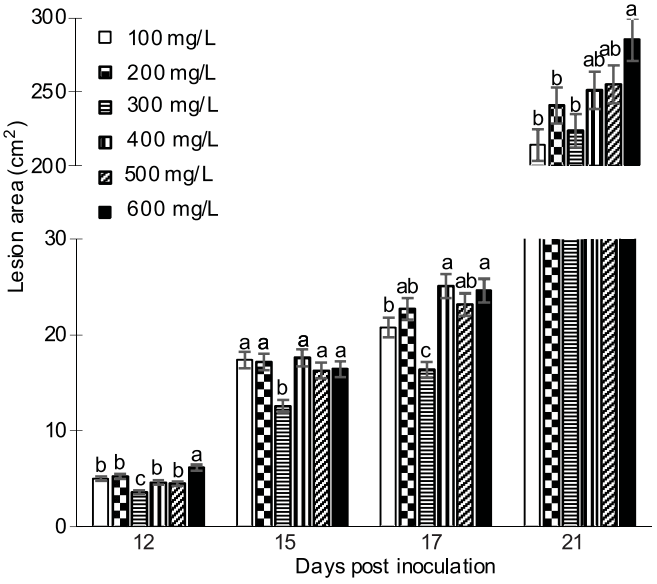

Fig. 1. Lesion area (cm^2) developed in cucumber (cv. Knossos) plants at 12, 13, 15, 17 and 21 days post inoculation with *Pseudoperonospora cubensis*, under different nitrogen doses (100-600 mg L^{-1} N) in soil. Values represent the mean (± SE) of measurements made on four independent leaves (in different plants) per treatment.

The leaf area increased with time in all N dose treatments (data not shown) with the polynomial curve best fitting the data. Symptoms of downy mildew were observed in all the plots. The total lesion area increased during the sampling dates in all treatments, reaching a maximum at the final sampling time (day 11).

The disease progress curves (cubic model) are described (R^2=0.99) as a consequence of different N doses and in more detail their equations were: $Y=-23.2+28.5x-7.4x^2+0.6x^3$ for the 100 mg L^{-1} N; $Y=-23.6+29.7x-7.9x^2+0.7x^3$ for the 200 mg L^{-1} N; $Y=-23.2+29.4x-7.1x^2+0.7x^3$ for the 300 mg L^{-1} N; $Y=-23.2+29.4x-7.9x^2+0.7x^3$ for the 400 mg L^{-1} N; $Y=-23.9+30.4x-8.3x^2+0.7x^3$ for the 500 mg L^{-1} N; and $Y=-26.5+34.1x-9.4x^2+0.8x^3$ for the 600 mg L^{-1} N treatments (data not shown). The greatest difference (up to 79.8 cm^2) on day 11 in terms of leaf area produced and lesion area covered by infection was found with the 300 mg L^{-1} N application which highlights the slower lesion extension when compared with leaf development.

Total accumulation of nutrients in the leaves and soil: All nutrient concentrations in cucumber leaves were in the sufficient range (Jones *et al.*, 1991). Nutrient analyses of the cucumber leaves and soil indicated differences in their accumulation (Tables 5 and 6). The Mn content decreased in the 100 mg L^{-1} N, while no differences were observed in higher N concentrations. There were no significant differences in the concentrations of Fe, Zn, Cu, Ca, Mg and K in the leaves (Table 5). The increase of N doses resulted in increased NH_4-N and NO_3-N concentrations

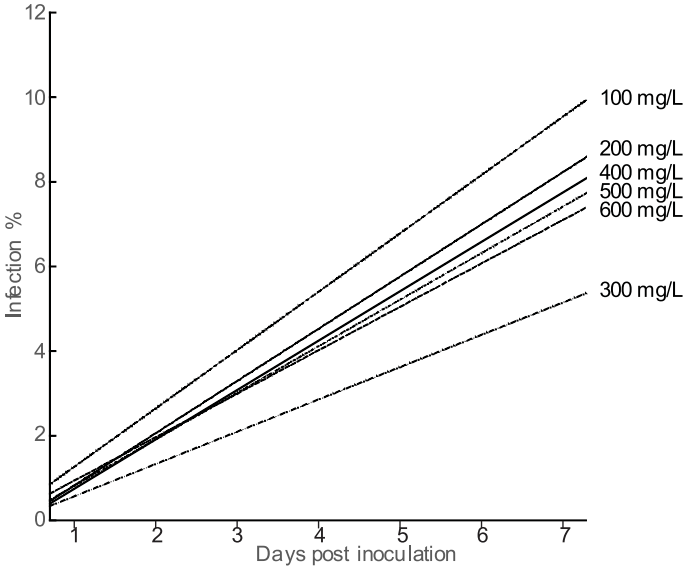

Fig. 2. Regression of infection percentage of cucumber plants, artificially infested with *Pseudoperonospora cubensis* at 11-17 days post inoculation

Table 3. Downy mildew development in greenhouse cucumber (cv. Knossos) plants fertilized with different nitrogen doses (100-600 mg L^{-1} N) in soil, as described by the percentage disease progress. Coefficients of determination (R^2), growth rates (slopes) and their corresponding standard errors are given, for simple linear regressions between day 11 and day 17 post artificial inoculation with *Pseudoperonospora cubensis*

N (mg L^{-1})	R^2 (%)	Slope	S.E
100	91.8	1.464	±0.103
200	95.7	1.333	±0.066
300	92.6	0.839	±0.056
400	94.9	1.276	±0.070
500	94.6	1.198	±0.067
600	96.3	1.111	±0.052

Table 4. Percentage of symptomatic leaf area of cucumber (cv. Knossos) leaves artificially infested with *Pseudoperonospora cubensis* at 21 days post inoculation and AUDPC under different N fertigation doses (100-600 mg L^{-1} N). Means within columns followed by different letters are significantly different according to Duncan (P=0.006)

N (mg L^{-1})	lesion/leaf area (%)	AUDPC
100	97.0 a	546.6e
200	86.3 ab	605.7d
300	73.7 c	535.7f
400	82.9 bc	634.5b
500	86.8 ab	634.3c
600	88.1 ab	701.8a

in cucumber leaves, while P concentration decreased with the application of 600 mg L^{-1} N. No correlation was observed among nitrogen treatments and the percentage infection, as well as between leaf nitrate or soil nitrate and downy mildew incidence (data not shown).

The results also indicated that NO_3-N gradually increased in soil as a consequence of the N application to the soil. For the macro elements in soil, the concentrations of K reduced with N > 200 mg L^{-1}. Thus, the application of higher nitrogen levels increased the concentration of NO_3-N in the soil for almost all the treatments (Table 6) which was directly reflected in increased NO_3-N in leaves. Thus, correlation analysis of the data showed that there was a high positive correlation between NH_4-N and NO_3-N in leaves (R^2=0.92), NH_4-N in leaves and NO_3-N in soil (R^2=0.87) as well as NO_3-N in leaves and NO_3-N in soil (R^2=0.93).

Discussion

This study showed that increased nitrogen supply in soil resulted in a progressive increase of NO_3-N and NH_4-N in the cucumber leaves which was directly related to the increased leaf area. Furthermore, the use of 300 mg L^{-1} N in the form of ammonium nitrate caused significant reduction of downy mildew symptoms. During the progression of the disease, the analysis of AUDPC, confirmed that the use of the above N concentration in the fertigation was the most effective treatment. Thus, in terms of AUDPC analysis, the maximum disease reduction was noted with the application of 300 mg L^{-1} N and 24% severity reduction was recoded. These results are similar to other reports on cereal plants where reduction in pathogen severity occurred after application of ammonium nitrogen fertilizer (Brennan, 1992). However, the suggestion was made that the form of nitrogen was the most important factor influencing "take all" disease. The significance of the nitrogen form in various plant-pathogen interactions has also been reported in other studies (Huber and Watson, 1974; Mandal *et al.*, 2008). Indeed, in the present study, this cannot be concluded as both nitrogen forms (NH_4-N and NO_3-N) were provided by ammonium nitrate addition.

The hypothesis that disease increased at the high nitrogen rates (400, 500 and 600 mg L^{-1}) due to increased N content in leaves was found to be untrue in the present study. This effect was also observed in low nitrogen (100 and 200 mg L^{-1}) treatments where the actual leaf N status was low. In the review articles of Walters and Bingham (2007) and Dordas (2008) it was reported that nutrients could affect infection by altering crop characteristics by changing microclimatic conditions. This is supported by the findings presented here, as the application of 100 mg L^{-1} N resulted in smaller leaves with the largest infection lesions when compared to the application of 300 mg L^{-1} N which resulted in medium sized leaves with smaller lesion areas (infected area). On application of 600 mg L^{-1} N, larger leaves with large lesions appeared, as shown by the slope values. The aforementioned researchers also suggested that the influence of nitrogen on downy mildew depended on the type of pathogen rather than the effect on microclimate or leaf nutrient status as has been found in other plant-pathogen pathosystems (Walters and Bingham, 2007). However, the above statement could not be validated in

Table 5. Nutrient concentration (macro- and micro-nutrients) in leaves of cucumber (cv. Knossos) plants, two days before the inoculation with *Pseudoperonospora cubensis,* grown in different nitrogen doses (100-600 mg L^{-1} N). Means (n=4) in column followed by the same letter are not significantly different, $P \leq 0.05$ according to Duncan's multiple range test

	Nutrients in leaves (mg g^{-1} dry weight)					
N-dose (mg L^{-1})	K	Ca	Mg	P	NH_4-N	NO_3-N
100	40.05 a	24.67 a	8.57 a	4.82ab	0.043 e	0.027 d
200	43.70 a	22.07 a	9.12 a	5.47 a	0.061 d	0.055 d
300	40.57 a	20.80 a	8.80 a	4.75 b	0.086 c	0.142 c
400	41.30 a	19.37 a	8.25 a	4.67 b	0.106 b	0.191 b
500	39.42 a	20.47 a	8.50 a	4.47bc	0.120ab	0.213 b
600	36.07 a	20.50 a	8.57 a	3.92 c	0.126 a	0.258 a
	Fe	Mn	Zn	Cu		
100	0.087 a	0.018 b	0.018 a	0.013 a		
200	0.088 a	0.036 a	0.018 a	0.013 a		
300	0.089 a	0.039 a	0.016 a	0.014 a		
400	0.084 a	0.036 a	0.017 a	0.011 a		
500	0.090 a	0.037 a	0.016 a	0.014 a		
600	0.083 a	0.035 a	0.016 a	0.013 a		

Table 6. Macronutrients in soil fertilized with different nitrogen doses (100-600 mg L^{-1} N), in cucumber (cv. Knossos) cultivation, two days before *P. cubensis* inoculation. Means (n=4) in column followed by the same letter are not significantly different, $P \leq 0.05$ according to Duncan's multiple range test

N-dose (mg L^{-1})	Nutrients in soil (mg g^{-1} dry weight)		
	K	NH_4-N	NO_3-N
100	0.242 a	0.072 a	0.021 e
200	0.184 b	0.051 b	0.030 de
300	0.190 b	0.067 a	0.069 d
400	0.196 b	0.058 ab	0.141 c
500	0.205 b	0.064 ab	0.190 b
600	0.187 b	0.060 ab	0.249 a

our study because as long as the pathogen's growth response curve did not follow the N supply. The past studies have suggested that nitrogen supply possibly affected disease as a result of an effect on the pathogens' requirements and possibly secondary host metabolites (defense associated compounds) that prevented fungal colonisation. Therefore, investigation of the secondary metabolism may be one area which needs further investigation.

This study suggests that in cucumber plants, low and very high levels of nitrogen inputs could promote disease development while intermediate concentrations (300 and 400 mg L^{-1} N) helped the plant to overcome the pathogen infection, resulting in a slower disease progress. There could thus be thresholds ranges of nitrogen supply which could be used beneficially, while too little or excess may have a detrimental impact. Furthermore, Tanaka *et al.* (2000) reported that the increase in the quantity of N in the nutrient solution had a positive effect on the nutrient content in the leaves in susceptible and resistant cucumber plants although the leaf N content was not closely correlated with the total lesion area of downy mildew. However, they used NO_3-N at three levels which was given to plants grown in hydroponics systems only and not in soil.

Nam *et al*. (2006) recently reported that elevated nitrogen concentrations in the fertilizer solution increased anthracnose severity in strawberry plants, in contrast to phosphorus and calcium, in non-circulation hydroponics systems. Nevertheless, previous studies have shown the reduced effect of nitrogen on plant diseases. Thus, Strengbom and Reich (2006) showed that although the increased N input did not cause N accumulation in the leaves of *Solidago rigida*, a decreased incidence of foliar symptoms was evidenced. Consequently, crop nutrition may influence disease development in a different way. Huber and Thompson (2007) also suggested that high nitrogen decreased the accumulation of defence related compounds in plants and hence increased the susceptibility to pathogens. In contrast, nitrogen deficiency results in weak plants which grow and senesce more quickly and thus are more susceptible to diseases. In the present study, this may be the mechanism operating as both low and high nitrogen levels led to an increase in plant susceptibility to diseases. David *et al.* (2003) stated that nitrogen fertilization had a positive effect on powdery mildew disease severity of *Hiemalis begonia*, primarily at the later production stages. In their study three levels of nitrogen and potassium were examined in a factorial experiment. In the same study it was also demonstrated that medium dose of N fertigation at 120 mg L^{-1}, their medium treatment concentration, could help limit powdery mildew incidence on begonia.

Although it is mostly believed that nitrogen generally increases crop susceptibility to diseases, the importance of the balance between various nutrients is also well established (Reuveni and Reuveni, 1998). Even though 300 mg L^{-1} of nitrogen is considered high, the normal rates that are used in Greece and Cyprus are between 150-300 mg L^{-1}. Thus in our experiments, the higher nitrogen concentration treatments increased lesion area of downy mildew when compared to the medium dose levels (300 mg L^{-1} of nitrogen). This is similar to what was suggested by Walters and Bingham (2007). They reported that nitrogen supply above recommended rates could increase lesion area which was caused by a combination of both biotrophic and necrotrophic pathogens. In conclusion, this study shows that 300 mg L^{-1} nitrogen for fertigation is a critical concentration value not only for the leaf size but also for the development of downy mildew disease. Thus, there are indications that lower N supplies could lead to increased downy mildew and subsequently have a major impact on final cucumber yields. Thus, the targeted nitrogen supply via ammonium nitrate fertilizer should be considered for the control of downy mildew in cucumber plants.

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