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Pathological response and biochemical changes in *Allium cepa* L. (bulb onions) infected with anthracnose-twister disease

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Abstract

Development of novel ways to address the losses and decreased production of onion in the Philippines due to diseases, has been of great interest since 1996. To achieve this, basic knowledge on the pathogenesis and host response are crucial in developing resistant varieties. Results generated from the pathogenicity test confirmed *Colletotrichum gloeosporioides* and *Gibberella moniliformis* are both pathogenic to the three onion cultivars tested (Yellow Granex, Red Creole and Shallot). Disease incidence and disease index were highest in Yellow Granex, followed by Shallot and Red Creole. Application of GA3 at 100 and 1000 µg/ml in the neck of onion plants produced severe twisting and elongation and microscopic examination showed that the cells are exceptionally long and wide as compared to the cells of the uninoculated plants. Biochemical analysis unveiled that protein content, amount of reducing and total sugars as well as phenols were low in all of the infected plants as compared to the uninoculated plants. A trend that shows a correlation between decreasing protein, sugar and phenols content and decreased resistance.

Key words – anthracnose-twister disease – *Colletotrichum gloeosporioides* – *Gibberella moniliformis* – Gibberellic acid

Introduction

Onion is one of the most important crops of the Philippines. Its production, however, is at a decreasing trend from 1996-2000 resulting in low supply and high cost in the local market as well as limited quantities of onion for export. One of the various factors that contribute to low production are pests and diseases. Among the diseases, the anthracnose-twister plays a very important role in its low productivity which at present is considered to be the most destructive disease of onion in the country (Alberto et al. 2002). It is characterized by severe twisting of leaves and neck elongation as well as necrosis of leaves. The causal organisms were identified as *Colletotrichum gloeosporioides* as the cause of the anthracnose symptom and *Gibberella moniliformis* which is suspected to be the cause of twisting and abnormal neck elongation due to excessive accumulation of gibberellins in onions. Alberto (2010) showed by species specific primers that the species was *C. gloeosporioides*, but since the species specific primers were not generated from the epitype strain (Cannon et al. 2008), this identity still needs verifying against the epitype by multigene analysis as recommended by Cai et al. (2009) and other recent publications.

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Information on the biological processes involved in *C. gloeosporioides* and *G. moniliformis* infection and the twisting effects in onions is at the moment non-existent. The absence of resistant varieties against the disease will also serve as an opportunity to investigate the weaknesses and the response of the onion plants to pathogen invasion and this will serve as one of the bases for genetic and molecular approach of managing the disease in the future. However, information as to the role of gibberellin on the neck elongation and twisting of leaves as well as on the pathological and biochemical response of onion after infection are still in its infancy, thus conducting this study.

Materials and Methods

Pathogenicity tests

Specimens infected with "anthracnose-twister" disease were collected in onion growing areas of San Jose City and Talavera, Nueva Ecija. The two target pathogens namely: *Colletotrichum gloeosporioides* and *Gibberella moniliformis* were isolated from the host plant, cultured, mass produced and tested its pathogenicity to onion cultivars where it was isolated. Prior to inoculation, the spore density of the two pathogens was standardized and 25 µl of the fungal suspension was dropped in three points of inoculation in each onion leaf. The host plants were observed for symptom development. KOCH's Postulates was satisfied when the pathogens were re-isolated from the host plants and re-checked its characteristics morphologically.

Role of GA3 on twisting and neck elongation of onion

GA3 assay was conducted by inoculating the following concentrations of GA3: 0.1, 1.0. 10.0, 100.0 and 1000.00 µg/plant into the neck of onion seedlings. Initial length of the leaves and neck were taken followed by observation and data collection at 4,8,16, 24, 48, 72 and 96 hrs after GA3 application. Data in cell dimension were also taken by cutting the inoculated part of the leaf and were preserved and examined microscopically for changes in cell width and cell length.

Pathological analyses

Ninety (90) day old potted seedlings of the three onion cultivars commonly grown in the Philippines namely: V1 - Yellow Granex (T1), V2 - Red Creole (T2), V3 – Shallot (T3) were used in the pathological and biochemical aspect of infection of *C. gloeosporioides*, *G. moniliformis* and *C. gloeosporioides* + *G. moniliformis*. Each treatment was divided into two sets as follows: I - Inoculated and UI – Uninoculated

Each set has six (6) potted seedlings i.e. one (1) for inoculated and one (1) for uninoculated, replicated three (3) times. The same number of potted seedlings makes the two other sets, thus, having a total number of eighteen (18) potted seedlings per group. The first group were inoculated with *C. gloeosporioides* and the two other groups were inoculated with *G. moniliformis* and *C. gloeosporioides* + *G. moniliformis*. Disease reaction on the leaves was scored on 0-9 scale at 7 days after inoculation. The following parameters were used to evaluate disease development and expressed as:

(a) $\%\ \text{Disease Incidence (\%DI)} = \dfrac{\text{No. of infected plants}}{\text{Total no. of plants}} \times 100$

(b) $\text{Disease Index} = \dfrac{\text{Sum of all numerical ratings} \times 100}{\text{Maximum grade (9)} \times \text{Number of leaves examined}}$

(c) Incubation period
(d) Size of spot (mm)
(e) Length of neck (cm)

The incubation period that was considered is the time (in days) between inoculations up to the first appearance of white-pin pricked spots on the leaves. A total of 3 leaves per plant were observed and the length and width of the spots per leaf (3) were recorded.

Biochemical Analyses

Leaf samples of inoculated and uninoculated onion leaves on the three sets of inoculated onion plants (a. *C. gloeosporioides* b. *G. moniliformis* c. *C. gloeosporioides* + *G. moniliformis*) were collected at 7 days after inoculation (DAI.). Samples were replicated three (3) times. The leaf samples were washed with sterile distilled water, air-dried and packed in three layered polythene bags and stored in deep freeze at $-l0^{o}C$ to be used later for the estimation of biochemical components.

Protein content was assessed following the Automated Calorimetry method. Total soluble sugars were estimated using the method described by Shaffer-Somogyi and the reducing sugars following the Quisumbing-Thomas method. High Performance Liquid Chromatography (HPLC) was used to determine the amount of phenols in the plant samples. Data obtained was analyzed using two-factorial CRD with 3 replications.

Results and Discussion

Pathogenicity Tests

Pathogenicity was confirmed after inoculating the leaves of 55 day old onion seedlings (cv. Yellow Granex, Red Creole and Shallot/Tanduyong) with the fungal suspensions of *Colletotrichum gloeosporioides* and *Gibberella moniliformis* with 100% disease incidence in all of the onion cultivars (Table 1). Disease indexes of 92.59% to 100% was observed in all three varieties inoculated with *C. gloeosporioides* + *G. moniliformis*, and 100% in plants inoculated with *C. gloeosporioides* while those onion plants inoculated with *G. moniliformis* alone showed varying degrees of infection ranging 70.37% in Red Creole, 92.59 in shallot to 100% in Yellow Granex (Table 2). All inoculated plants showed anthracnose symptom on the leaf, twisting of leaves and neck elongation 5 days after inoculation (Fig.1). Seedlings inoculated with sterile distilled water remain unaffected. The two pathogens were re-isolated from the diseased onion plants to satisfy Koch's postulates (Fig.2).

Table 1 Disease incidence (%) of *C. gloeosporioides* and *G. moniliformis* in three onion cultivars.

Onion Cultivars	**Pathogens***			
	C.g	**G.m**	**C.g + G.m**	**UI****
Yellow Granex	100	100	100	0
Red Creole	100	100	100	0
Shallot	100	100	100	0

* C.g = *Colletotrichum gloeosporioides*; G.m.= *Gibberella moniliformis*; C+G = *C. gloeosporioides* + *G. moniliformis*
** Uninoculated

Table 2 Disease index (%) of *C. gloeosporioides* and *G. moniliformis* in three onion cultivars.

Onion Cultivars	**Pathogens***			
	C.g	**G.m**	**C.g + G.m**	**UI****
Yellow Granex	100.00	100.00	100.00	0.00
Red Creole	100.00	70.37	92.59	0.00
Shallot	100.00	92.59	100.00	0.00

* C.g = *Colletotrichum gloeosporioides*; G.m.= *Gibberella moniliformis*; C+G = *C. gloeosporioides* + *G. moniliformis*
** Uninoculated

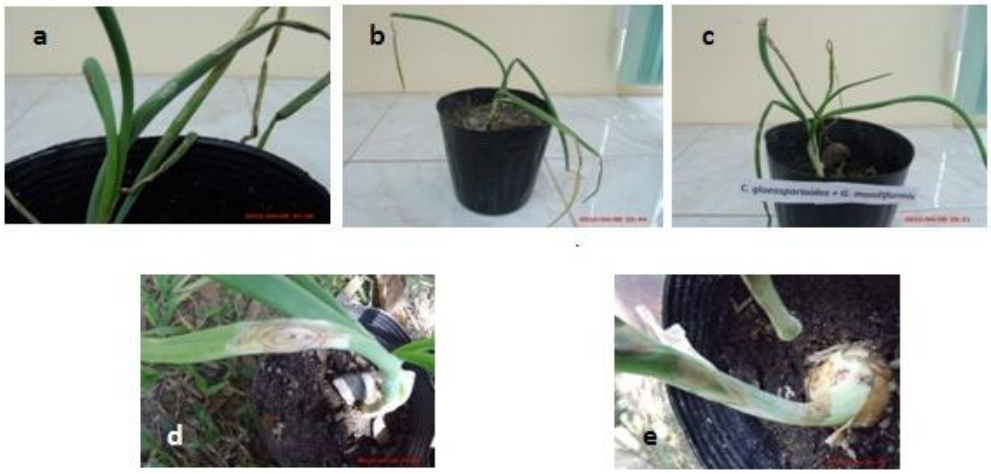

Fig. 1 – Pathogenicity test of *C. gloeosporioides*, and *G. moniliformis i*n Yellow Granex a, C. *gloeosporioides* b, *G. moniliformis* c, *C. gloeosporioides* + *G. moniliformis* d, typical antracnose symptom e. elongated neck in onion infected with both C. *gloeosporioides* + *G. moniliformis*

Fig. 2 – a, *G. moniliformis* b, *C. gloeosporioides* c, *C. gloeosporioides* and *G. moniliformis* re-isolated from previously infected onion plants

Role of GA3 on Twisting and Neck Elongation of Onion

This study showed the effects of GA3 on the growth and development of onion plants. Two weeks after application of GA3, neck elongation and twisting of leaves were very evident in onion plants applied with 100 µg and 1000 µg of GA3 (Table 3). Similarly, the longest leaves and neck were observed in the same treatments (Fig. 3). Microscopic examination of the tissues treated with GA3 exhibited longer and wider cells than cells of the uninoculated plants (Fig.4) that triggers abnormal neck elongation and twisting. The plants treated with high concentration of GA3 mimicked the symptoms exhibited by the onion plants infected by the *G. moniliformis* in the field.

Table 3 Effect of GA3 in onion seedlings two weeks after application

Main	Length (cm)		Twisting	Cell Size (u)	
Treatments (ug)	Leaves	Neck	Effect*	Length	Width
T1 (0.1)	27.85c	2.50c	0	409.56d	46.12d
T2 (1.0)	28.77b	2.70c	0	414.05c	54.61c
T3 (10.0)	29.10b	3.33b	1	425.71c	57.34c
T4 (100.0)	29.16b	3.57b	3	529.27b	62.60b
T5 (1000.0)	31.13a	3.66a	3	558.47a	73.82a
T6 Control (-)	27.79c	2.43c	0	407.13d	44.29d
T7 Control (+)	30.95a	3.64a	3	561.37a	72.84a

*Rating Scale: 0-Plants standing, neck not twisted; 1-Plants standing with slight neck twisting; 2-Plants with twisted neck, plants still erect; 3-Plants with severe neck twisting, and leaves down to ground

Fig. 3 – Effect of GA3 in onions two weeks after application. a, Control (-) b, 1.00 µg, c, 10.00 µg d, 100.00 µg e, 1000.00 µg

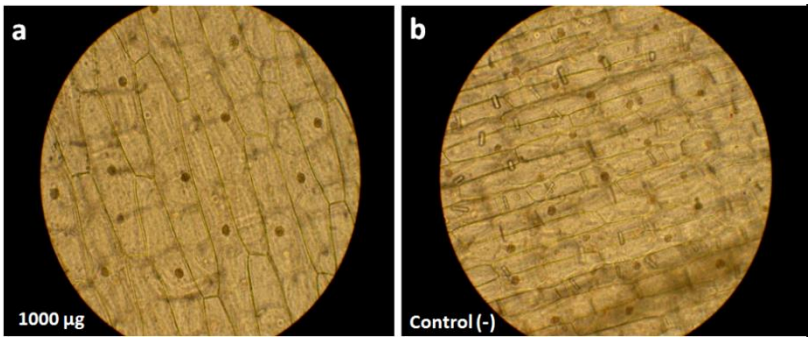

Fig. 4 – Cells in neck of onion seedlings treated with **a.** 1000.00 ug of GA3 and **b.** sterile distilled water Gibberellin, a known growth hormone in plants and a mutagen in excess amount stimulates the growth and development of the cells resulting to uneven growth of cells in the affected tissues. The results were consistent with the findings of Brian and Hemming (1955) in pea where progressive increase in the length of internodes of pea was observed in plants treated with higher concentration of GA3. Moreover, it was observed that there was a period of enhanced growth in the plants treated with higher concentration of GA3 (100-1000.00 µg).

Pathological Analysis

All the three onion cultivars inoculated with the two pathogens and their combination were all infected by the disease (100% DI). The uninoculated plants remain uninfected (Table 4). The Disease in Yellow Granex was so severe such that it got the highest disease index (100%) upon infection of the three pathogens. This was followed by Shallot with 100% disease index when inoculated with *C. gloeosporioides* and when it was combined with *G. moniliformis*. Red Creole succumb only from the disease upon infection by the two pathogens but shows lower disease index to *G. moniliformis* infection (62.19%). However, all the three onion cultivars inoculated with both pathogens have 100% disease index. The *G. moniliformis* inoculated plants showed lower disease index most especially in Red Creole and Shallot. However, disease index was higher in Yellow Granex (Table 5).

Table 4 Disease incidence (%) in three onion cultivars infected with *C. gloeosporioides* and *G. moniliformis* 5 days after inoculation

Pathogens	**Onion Cultivars**		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	100.00ns	100.00ns	100.00ns
G. moniliformis	100.00	100.00	100.00
C.g + G.m	100.00	100.00	100.00
Untreated Control	0.00	0.00	0.00

Table 5 Disease index (%) in three onion cultivars infected with *C. gloeosporioides and G. moniliformis* 5 days after inoculation

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	100.00ns	70.37b	100.00a
G. moniliformis	100.00	62.19c	85.18b
C.g + G.m	100.00	100.00a	100.00a
Untreated Control	0.00	0.00d	0.00c

The shortest incubation period for the pathogens to show symptoms was observed in Yellow Granex and Shallot, ranging from one day to 1.33 days especially so when the plants were inoculated with the two pathogens. It took only one day for both pathogens to show their symptoms in the plants as compared to Red Creole that needs at least 1.66 days to show its symptoms and much longer (2 days) when these two pathogens were inoculated separately in onion plants (Table 6). This indicates that the Red Creole has some resistance mechanism working against the two pathogens that delays its infection process.

Table 6 Incubation period (days) of *C. gloeosporioides* and *G. moniliformis* in three onion cultivars.

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	1.33a	2.00a	1.33a
G. moniliformis	1.33a	2.00a	1.33a
C.g + G.m	1.00a	1.66b	1.00a
Untreated Control	0.00b	0.00c	0.00b

The largest size of the leaf spot due to *C. gloeosporioides* was observed in Yellow Granex (3.33cm) and Shallot (3.30cm) and smaller in Red Creole (1.73cm). The spot tend to become bigger (2.87cm to 3.40cm) when the two pathogens infect the plants (Table 7). Small spots were observed in *G. moniliformis* infected plants for reason that the pathogen is an endophytic fungi, it does not destroy plant tissues but instead colonized the internal structures of the plant tissues.

Table 7 Length of spot (cm) of *C. gloeosporioides* and *G. moniliformis* in three onion cultivars 5 days after inoculation.

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	3.33a	1.73b	3.30a
G. moniliformis	0.25b	0.23c	0.24c
C.g + G.m	3.40a	2.87a	3.40a
Untreated Control	0.00b	0.00c	0.00c

The longest neck and twisting of leaves were very evident in Yellow Granex inoculated with the combination of *C. gloeosporioides* and *G. moniliformis* (7.87cm); this was followed by Yellow Granex inoculated only with *G. moniliformis* (4.73cm). Similar results were observed in Red Creole though all of the onion cultivars become twisted five days after inoculation. Shorter neck was noted in Shallot owing to the nature of the plant which is normally smaller than the two onion cultivars (Table 8).

Disease was so severe in all of the three onion cultivars inoculated with the combination of *C. gloeosporioides* and *G. moniliformis* (Table 9 and Fig.5). Twisting was so severe such that the plants die and fall to the ground in just 5 days. But in Red Creole, severe twisting of leaves and neck elongation only occurred 6 to 7 days after inoculation, a consistent characteristic of the plant

Table 8 Length of the neck (cm) of three onion cultivars infected with *C. gloeosporioides* and *G. moniliformis* 5 days after inoculation

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	3.57c	3.53c	2.20c
G. moniliformis	4.73b	5.67b	3.70b
C.g + G.m	7.87a	7.20a	4.27a
Untreated Control	3.50c	3.40c	2.11c

Table 9 Twisting effect of *C. gloeosporioides* and *G. moniliformis* on three onion varieties 5 days after inoculation

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	0**	0	0
G. moniliformis	9	7	9
C. g + G. m	9	9	9
Untreated Control	0	0	0

** 0 = no symptoms
1 = slight twisting from the neck
3 = slight twisting from the neck with yellow green discoloration of the leaves
5 = twisting of the leaves with slight neck elongation
7 = twisting of leaves with severe neck elongation
9 = twisting of leaves with elongated neck and leaves down to soil surface

Fig 5 – *C. gloeosporioides + G. moniliformis* in Yellow Granex, Red Creole and Shallot

exhibiting delay of infection or partial resistance against the two pathogens. Seedlings sprayed with sterile distilled water remain uninfected, grows and developed to full maturity to produce bulb.

Biochemical Analysis

Biochemical analysis showed that the protein content of uninoculated onion plants was higher than the inoculated plants (Table 10). The three onion cultivars inoculated with *C. gloeosporioides* has the lowest protein content, i.e. 10.68%, 9.58% and 13.74% for Yellow Granex, Red Creole and Shallot, respectively followed by plants inoculated with the combination of *C. gloeosporioides + G. moniliformis* with 14.81% for Yellow Granex, 16.94% for Red Creole and 20.61% for Shallot. Among the three inoculated cultivars, protein content was highest in Shallot and lowest in Yellow Granex. Similarly, the total soluble sugars and reducing sugars were also higher in the uninoculated onion plants and low in the three inoculated onion plants (Tables 11 and 12).

Table 10 – Protein content (%) in the leaves of onions infected with *C. gloeosporioides* and *G. moniliformis*

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	10.68b	9.58c	13.74d
G. moniliformis	15.68a	15.49b	24.67b
C.g + G.m	14.81c	16.94a	20.61c
Untreated Control	16.12a	17.23a	26.15a

Table 11 Amount of total soluble sugar (%) in the leaves of onions infected with *C. gloeosporioides* and *G. moniliformis.*

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	3.10b	1.68b	5.72c
G. moniliformis	2.42c	1.85b	6.15b
C.g + G.m	2.18c	1.26c	3.03d
Untreated Control	3.35a	2.30a	6.74a

Table 12 Amount of reducing sugar (%) in the leaves of onions infected with *C. gloeosporioides* and *G. moniliformis*.

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	0.81b	0.48b	1.50b
G. moniliformis	0.79b	0.44b	1.24b
C.g + G.m	0.76b	0.43b	0.63c
Untreated Control	1.76a	1.54a	2.29a

It appears that there is a general trend towards decreasing resistance in host tissues with decreasing total sugar content. Decrease in sugar levels after infection could be due to rapid hydrolysis of sugars during pathogenesis through enzymes secreted by the pathogen (Jaypal & Mahadevan, 1968). Also, the increased in reducing sugar content correlates with resistance, as well as the decrease in content after infection (Yadav et al, 1994).

On the otherhand, very low levels of phenol on the leaves were detected in all plant samples, including the uninoculated (Table 13). These results explains the low level of resistance and susceptibility of onion plants to foliar infection of different fungal pathogens because the accumulation of total phenols is usually higher in resistant than susceptible genotypes (Arora and Wagle, 1985; Bashan, 1986; Mishra et al. 2008). It also strengthens the findings of Alberto et.al. (2002) in onions where no resistant cultivar was found when artificially inoculated by *C. gloeosporioides* under field conditions.

Table 13 Total phenols (%) in the leaves of onions infected with *C. gloeosporioides* and *G. moniliformis*

Pathogens	Onion Cultivars		
	Yellow Granex	Red Creole	Shallot
C. gloeosporioides	0.250	0.360	0.038
G. moniliformis	0.032	0.130	0.040
C.g + G.m	0.250	0.009	0.023
Untreated Control	0.063	0.073	0.085

Summary and Conclusion

The fungal plant pathogens isolated from the anthracnose-twister infected onion plants from the field were confirmed to be the causal organisms of the disease. The GA3 was validated to play a very important role in the disease development as it mimics the symptoms of the disease upon application in the neck of the onion seedlings.

The three onion cultivars succumbed from infection of *C. gloeosporioides* and *G. moniliformis.* Among the three, however, Yellow Granex infected with *C. gloeosporioides* got the highest disease index, incidence, shorter incubation period and largest spots on leaves. It was also found to be most susceptible against *G. moniliformis* as shown by severe twisting of leaves and neck elongation. The disease progresses very fast and become very severe in Yellow Granex when exposed to combination of *C. gloeosporioides + G. moniliformis.* Biochemical analysis showed the low amount of total sugars, reducing sugars and protein in the infected onion cultivars as compared to higher amount of these compounds in the uninfected onion cultivars. Traces of phenols and the presence of these chemical compounds in lower amount makes the plant more susceptible to infection by the pathogens.

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