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THIOPHANATE METHYL RESISTANCE AMONG *Colletotrichum gloeosporioides* POPULATIONS IN SRI LANKA CAUSING ONION ANTHRACNOSE

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ABSTRACT

Anthracnose caused by *Colletotrichum gloeosporioides* is among the most important diseases of onion in Sri Lanka. Thiophanate methyl has been widely used in the control of anthracnose disease in onion in many growing areas in Sri Lanka. Poor control of anthracnose was reported following the application of thiophanate methyl. Therefore, this study was undertaken to determine the sensitivity of *C. gloeosporioides* to thiophanate methyl. Seven isolates of *C. gloeosporioides* were grown on PDA amended with 1, 10, 50, 100, 250 and 500 µg/ml thiophanate methyl and mycelial growth was measured. The isolates collected from Matale district were resistant to thiophanate methyl. No thiophanate methyl resistant isolates were recovered from Anuradhapura district. In greenhouse experiment, thiophanate methyl did not control the diseases caused by resistant isolates, whereas, disease control was achieved for the sensitive isolates. The fitness of resistant isolates was compared with that of sensitive isolates. Results revealed that the growth parameters of resistant isolates were not impaired by thiophanate methyl. Continuous selection pressure was maintained to investigate how fast isolates of *C. gloeosporioides* adapt to thiophanate methyl. The data revealed that *C. gloeosporioides* rapidly adapted to high concentrations of the fungicides.

KEYWORDS: Anthracnose, Colletotrichum gloeosporioides, onion, Resistance isolates, Thiophanate methyl

INTRODUCTION

Big onion is an economically important crop grown almost year round in Matale district and some parts of Anuradhapura district. The incidence of anthracnose caused by *Colletotrichum gloeosporioides* (penz) is wide spread and in some years significant yield losses occur especially during the *maha* season (Weeraratne, 1996; 1997). The disease symptoms include severe leaf curling,

twisting, chlorosis and abnormal elongation of psuedostem followed by bulb rotting.

The symptoms on foliage and flower stalks are characterized by the development of lesions that eventually coalesce to form large infected areas. Among various methods, fungicides form an important tool for managing anthracnose disease, because farmers believe that the chemical fungicides are more effective than the other methods. The fungicide thiophanate methyl belonging to benzimidazole group is in current use for the management of anthracnose in onion (Rajapaksha *et al.*, 2001) in Sri Lanka. This is a systemic fungicide which binds to beta tubulin and prevents the formation of microtubules, leading to the disruption of chromosome migration during fungi mitosis and meiosis (Davidse, 1986).

In recent years, losses due to anthracnose in onion have sometimes been severe despite the use of thiophanate methyl according to the onion farmers. One possible explanation for failure of the fungicides to control disease is the insensitivity of *C. gloeosporioides* to thiophanate methyl. Although the chemical fungicides effectively suppress and control a wide variety of plant diseases their continuous use, particularly the systemic fungicides, could reduce their effectiveness due to the development of resistance which has become a challenging problem in the management of crop diseases. The development of resistance towards fungicides has threatened the performance of some highly potent commercial fungicides (Brent, 1995). Resistant *C. gloeosporioides* isolates have been reported elsewhere in the world and it has led to failure of disease control using benzimidazole fungicides (Kumar *et al.*, 2007, Kim *et al.*, 2007). There is evidence that this loss of effectiveness is linked to a single point mutation in the gene encoding the fungicide targeted protein β - tubulin (Chung *et al.*, 2006, Ishii *et al.*, 1998, Nalumpang *et al.*, 2010).

To effectively control anthracnose disease in onion, it is necessary to determine the sensitivity of isolates of *C. gloeosporioides* to thiophanate methyl. Thus, the main objective of this study was to examine the sensitivity of *C. gloeosporioides* obtained from onion cultivations in Matale and Anuradhapura districts to thiophanate methyl. Furthermore, the impact of fitness components

of resistant isolates and ability of sensitive isolates to cause disease under induce fungicide pressure were also determined.

MATERIALS AND METHODS

Collection of Isolates

Onion leaves showing anthracnose symptoms were collected from seven locations of which four were from major onion growing regions of Matale district and three were from Anuradhapura district. The test isolate was collected from the Plant Pathology research field at Field Crops Research and Development Institute, Mahailluppallama where, no fungicides were applied (Table 1). Isolation was done using plant segments grown on Potato Dextrose Agar medium (PDA) at 250C and purified by single spore isolation method (Rangaswami and Mahadeven, 1999). The colour of the colony, shape and the colour of the conidia were examined with those of Colletotrichum gloeosporioides described in CMI description (Sutton, 1980). Thus, confirming its identification and maintained on PDA for further studies.

Table 1. Details of *C. gloeosporioides* isolates collected

Isolate	Location(Place of collection)	District
Cg 1	Dambulla	Matale
Cg 2	Galewela	Matale
Cg 3	Naula	Matale
Cg 4	Kibissa	Matale
Cg 5	Thambuththegama	Anuradhpura
Cg 6	Thalawa	Anuradhapura
Cg 7	Mahailluppallama	Anuradhapura

Determination of *C. gloeosporioides* sensitivity to thiophanate methyl

Variability in fungicidal sensitivity among the isolates was evaluated by poisoned food technique under in vitro conditions (Nene and Thapliyal, 1993). Mycelial disks of the pathogen removed from the margins of 10 day old culture were transferred to PDA amended with thiophanate methyl at concentrations of 1, 10, 50, 100, 250 and 500 µg/ml. Petriplates without fungicide were used as controls.

For each isolate, four replicates per concentration were used. These inoculated petriplates were incubated at 25°C. The experiment was conducted twice. After ten days of incubation two perpendicular colony diameters were measured on the bottom of the each plate. The two colony diameters were averaged for each plate and a mean diameter was calculated. The per cent inhibition in mycelial growth was calculated by the following formula.

$$\% \text{ Inhibitation of MG} = \frac{\text{CD Control - CD Treatment}}{\text{CD Control}} \text{ X } 100$$

ED50 value, the effective dose capable of reducing 50% of the colony growth was determined for each isolate.

Efficacy of thiophanate methyl in controlling anthracnose caused by sensitive and resistant isolates

The same isolates were evaluated in a greenhouse experiment using onion variety Rampur red. One month old seedlings for bulb crop and sprouted onion bulbs for seed crop were raised separately in pots containing sterilized soil. Two month after planting, both bulb crop and seed crop were sprayed to runoff with thiophanate methyl at the rate of 6g/10 liter of water. Control plants were sprayed with sterile distilled water. Two days later, 10 pots of each fungicide treated and control plants were inoculated with each isolate by spraying a 10 ml portion of a spore suspension (105 conidia/ml). Then the inoculated plants were transferred to a moist chamber at 25°C for 20 days. The experiment was repeated for three times. Percent leaf surface area with disease symptoms was estimated on individual plants with 0-7 scale as 0 = no symptoms, 1 = < 5 % leaf area affected, 3 = 6 –25% leaf area affected, 5 = 26 – 50 % leaf area affected, 7 = > 50% leaf area affected.

Disease severity index for each isolate was calculated for thiophanate methyl treated and untreated plants.

$$DS\% = \frac{\sum W \times N_s}{T \times N_m} \times 100$$

DS = Disease severity
$\sum W$ = Number of affected plants
N_s = Severity scale
T = Total number of observations
N_m = Maximum scale number (4)

Effect of thiophanate methyl on fitness components of C. gloeosporioides sensitive and resistant isolates

Fitness components of sensitive (Cg7) and resistant isolates (Cg1) were assessed and compared.

Spore germination

A suspension of conidia of *C. gloeosporioides* from the ten day old culture tubes were used in the experiment. For preparing the suspension, sterile distilled water was added to the culture tubes (slants) and gently scraped to remove mycelium. Conidia suspension was then filtered through double layer of muslin cloth under aseptic conditions to remove the hyphae and agar. The concentration of the suspension was adjusted to 1x 105 conidia/ml with the help of a haemocytometer. Drops (0.02 ml) of suspension were added to cavity slides. These slides were placed in petriplates lined with moist filter paper and incubated at 25°C for 24 h. The germination of spores was recorded. A spore is arbitrarily defined as germinated if the length of the germ tube exceeds half the smaller diameter of the spore. Per cent spore germination was calculated by the following formula.

$$\text{Spore germination \%} = \frac{\text{Number of Conidia Germinated}}{\text{50 Conidia}} \text{ X } 100$$

Sporulation

To measure the sporulation, the isolates were cultured on PDA at 25°C for 10 days and the conidia produced were harvested by adding 10 ml of distilled sterilized water in each plate. Each spore suspension was filtered through double layer of muslin cloth. The spore concentration in the suspension was measured with the aid of a haemocytometer and expressed as the number of conidia per cm^2

of the PDA culture. Four replicate droplets were counted for each plate and four plates were used for isolate.

Mycelial growth

For mycelial growth test, a mycelial plug (5 mm in diameter) of each selected isolate was transferred to the centre of a petriplate containing fungicide free PDA. After incubation of plates at 25°C for 10 days, colony diameter was measured.

Virulence

Virulence of resistant and sensitive isolates was determined by measuring length of the lesions on onion plants grown in pots. Conidia suspension was prepared from 10 days old culture tubes Plants were inoculated with conidia suspension (1x105 conidia/ml) evenly on to the 2 months old onion plants using hand sprayer. Three replicates for each isolate were used and incubated in a moist chamber at 25°C for 20 days.

In vitro derived resistance isolates

Onion seedlings were grown in plastic pots containing sterilized soil. The recommended dosage of thiophanate methyl (Topsin –M 70% WP, Nippon Soda Co. Ltd., Japan) is 6g /10 l of water. Based on this dosage three different rates of the fungicide 8, 10 and 12 g/ 10 l of water were applied after two months of planting. Fungicides were applied using a knapsack sprayer simulating field condition. Two days later, fungicide applied plants were inoculated with spore suspension of Cg6 sensitive isolate as described earlier (The first generation of Cg7 isolate did not produce enough conidia to start this experiment). Inoculated plants were transferred to a moist chamber at 25°C. Twenty days after inoculation, percent leaf surface area with disease symptoms was measured and disease severity was assessed as described earlier. These leaves were removed and the pathogen was re-isolated from the diseased leaves and used for the inoculation of a new set of fungicide treated onion seedlings. The procedure was repeated in seven consecutive times.

Data analysis

All data for colony diameter, DSI and fitness component were subjected to the analysis of variance (ANOVA). Analysis of percentage data were based on arcsine transformed percentage values. Mean values were compared using the Duncan's New Multiple Range Test (DNMRT). All the statistical analysis was done by SAS (Statistical Analysis System; SAS institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Isolates

Studies on morphological characteristics of fungal cultures showed that aerial mycelium of the colonies of all isolates is white and grey in colour on the upper surface and grey to black colour on the lower surface of the culture plate. They form cylindrical conidia with obtuse ends, aseptate and hyaline. These morphological characteristics were identical with that of *C. gloeosporioides* referred by Sutton (1980).

Determination of *C. gloeosporioides* sensitivity to thiophanate methyl

Thiophanate methyl was inhibitory to mycelial growth of three isolates viz. Cg5, Cg6 and Cg7 (Table 2). Mycelial growth of Cg1, Cg2, Cg3, Cg4 isolates showed no apparent reduction in colony diameter over the complete range of fungicide concentrations tested. The interaction between isolate and concentration was significant, indicating that there was a differential response of *C. gloeosporioides* isolates to thiophanate methyl concentration.

Four isolates of *C. gloeosporioides* had ED50 values of > 500 ?g/ml for thiophanate methyl and were grouped as highly resistant, whereas three isolates had ED50 values between 1 to 10 ?g/ml and classified as sensitive. All of the isolates obtained from Matale district were resistant to thiophanate methyl. There were significant differences in percentage inhibition of mycelial growth among sensitive isolates at each concentration. No thiophanate methyl resistant isolate was recovered in Anuradhapura district even though these fields had been treated with thiophanate methyl. The occurrence of thiophanate methyl resistant isolates in Matale district may be due to widespread use of thiophanate methyl. The onion

crop is frequently grown in Matale district than Anuradhapura district. Matale district also has a higher annual rainfall than the Anuradhapura district, which provides conditions favorable for rapid production of *C. gloeosporioides*. Thus, the potentially greater pathogen populations exposed to thiophanate methyl within a given field in Matale district may explain the development of thiophanate methyl resistant in Matale than Anuradhapura district.

Table 2. The effect of various concentration of thiophanate methyl on the percentage inhibition of mycelial growth and ED50 interval of different isolates

Isolate	Percentage inhibition of mycelial growth in different concentration (µg/ml) of fungicides						
	1	10	50	100	250	500	ED_{50} (µg/ml)
Cg 1	0	0	0	0	6.32	7.33	> 500
Cg 2	0	0	0	0	7.00	7.45	>500
Cg 3	0	0	0	0	7.23	8.00	>500
Cg 4	0	0	0	0	6.45	7.33	>500
Cg 5	44.44	66.66	88.88	97.77	100	100	1-10
Cg 6	46.66	63.33	90	95.55	100	100	1-10
Cg 7	41.11	72.22	91.1	100	100	100	1-10

The isolate recovered from plant pathology fields at Mahailluppallama was sensitive to thiophanate methyl, since none of the plants from which isolates were collected had been treated with thiophanate methyl. Benzimidazole resistance in *C. gloeosporioides* has previously been reported by several researchers (Chung, 2006, Kim *et al.*, 2007, Kumar *et al.*, 2007, Nalumpang *et al*, 2010).

Efficacy of thiophanate methyl towards sensitive and resistant isolates

When thiophanate methyl was not applied resistant and sensitive isolates caused similar lesions (no statistical difference) on leaves (Figure 1).

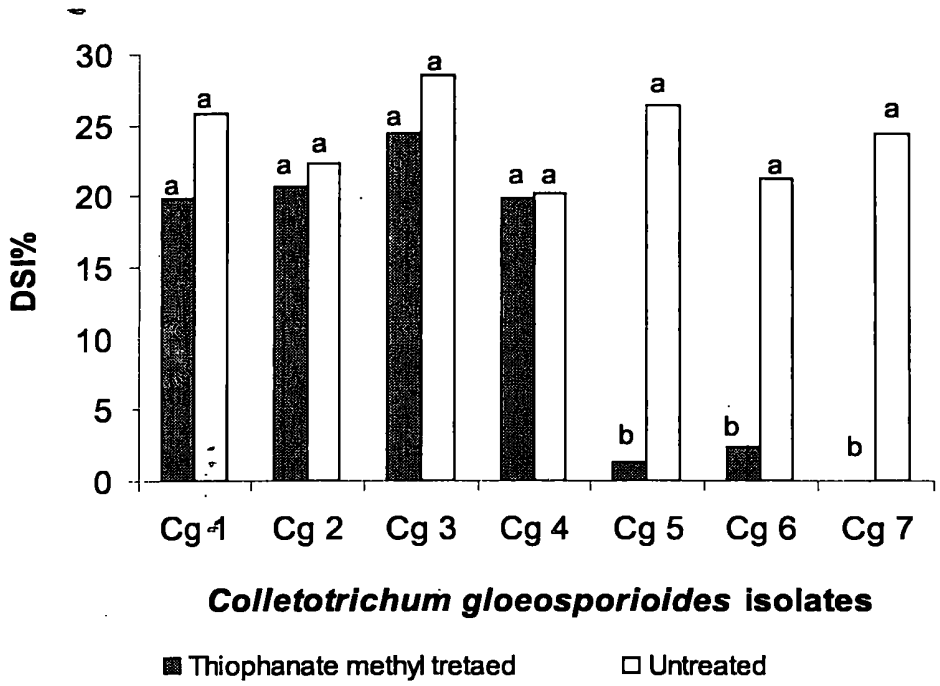

Figure 1. DSI of thiophanate methyl treated and untreated plants inoculated with resistant (Cg1, Cg2, Cg3, Cg4) or sensitive (Cg5, Cg6, Cg7) isolates

However, when thiophanate methyl was applied on the plants before inoculation with C. gloeosporioides, sensitive isolates caused significantly smaller lesions than the resistant isolates. Lesions were not observed on plants inoculated with sensitive isolate Cg7. In plants inoculated with sensitive isolates, Cg 5, Cg6 and Cg7, there was a significant difference in DSI between fungicide treated and untreated plants. In pots inoculated with resistant isolates, Cg1, Cg2, Cg3 and Cg4, DSI was lower in sprayed pots than unsprayed pots and this difference was not significant indicating that for resistant isolates, thiophanate methyl did not significantly control disease, whereas, disease control was achieved for the sensitive isolates. Waller (1992) reported that fungicide resistance appeared when benzimidazoles were widely used to control Colletotrichum diseases in a number of crop species.

Effect of thiophanate methyl on fitness components of sensitive and resistant isolates of C. gloeosporioides

There were no significant differences in mycelial growth, spore germination

and sporulation between resistant and sensitive isolates (Table 3). The results obtained have shown that the growth parameters of resistant isolates were not impaired by thiophanate methyl. The virulence of resistant and sensitive isolates on onion plants was examined. No significant difference in the ability to cause disease was observed between resistant and sensitive isolates. These data suggest that *C. gloeosporioides* resistant isolates appear to be virulent as the sensitive isolates. Therefore, resistant isolates would increase to a dominant level causing difficulty in controlling anthracnose. The occurrence of resistant isolates that are as fit as sensitive isolates has been observed with most genera of fungi in which benzimidazole resistance has been documented (De Waard *et al.*, 1993; Staub 1991).

Table 3. Comparison of spore germination, colony diameter, sporulation and lesion length between sensitive and resistant isolates

Isolate	Spore germination (%)	Colony diameter (cm)	Sporulation (no. of conidia / cm^2 of the PDA culture)	Lesion length (cm)
Cg 1	92	5.61	19	2.11
Cg 7	90	5.32	17	2.03
	ns	ns	ns	ns

In vitro derived resistance

All seven generations of the sensitive isolate, Cg6 showed uniform growth on the untreated control plants (Figure 2).

The first to fourth generations were susceptible to all concentrations of the fungicides. The fifth, sixth and seventh generations showed significantly higher DSI indicating adaptation to the fungicide. Continuous application of fungicides simulate a permanent selection pressure on the fungal population, which may enforce development of resistance and *C. gloeosporioides* pathogen rapidly adopted to high concentrations of the fungicides. The fifth generation of the fungus was able to grow on leaves containing high concentration of thiophanate methyl. *C. gloeosporioides* isolates resistant to thiophanate methyl were found in plant house on onion within 21 months after the first use of thiophanate methyl. It showed that continuous application enhanced resistant development in Colletotrichum spp against benzimidazole fungicides as reported by Sariah,

(1989) and LaMondia, (2001).

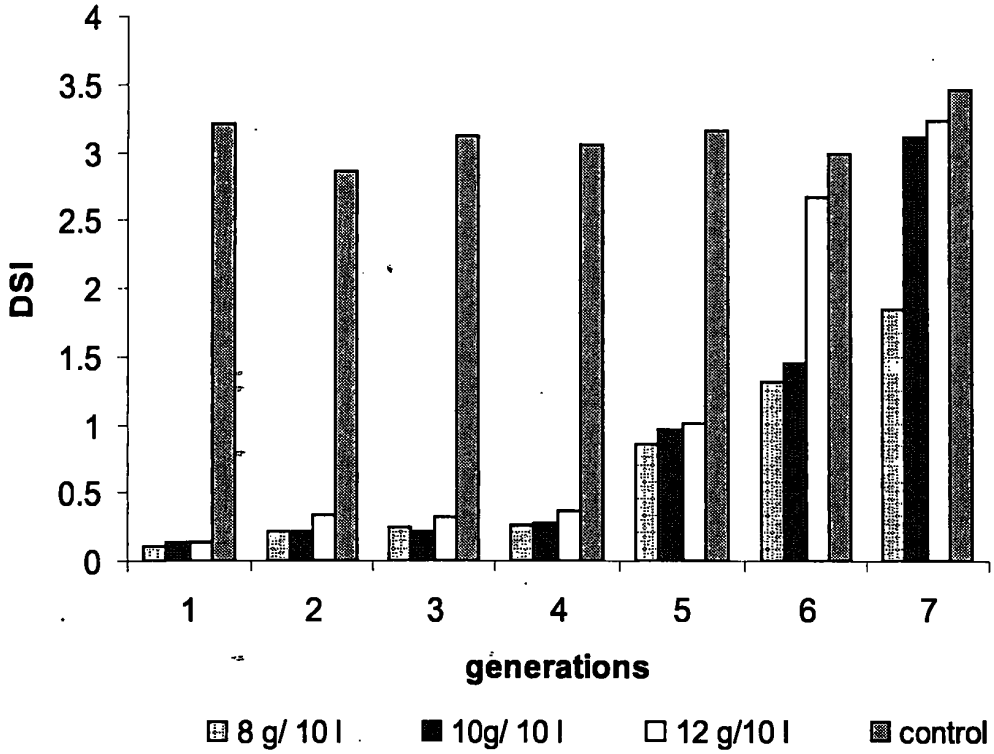

Figure 2. Disease severity induced by seven consecutive generations of *C. gloeosporioides* isolates raised on fungicide treated onion plants

A higher concentration is used in practice in the field by many farmers. They may also be indicative that fungicide resistant to thiophanate methyl in *C.gloeosporioides* isolates in Anuradhapura district is on the rise. Thus repeated use of high dose of thiophanate methyl should be avoided and development of resistance to this fungicide should be closely monitored in Anuradhapura district.

CONCLUSIONS

The data presented here demonstrate that there is a risk of *C. gloeosporioides* developing resistance to thiophanate methyl in the field over time. No other reports, except from Matale, of control failure after applying thiophanate methyl fungicides are known, and these fungicides are still providing excellent control of anthracnose in onion in other parts of Sri Lanka. However, any cases of poor disease control following application of thiophanate methyl fungicides will now need to be checked to determine if this is due to the build up of a thiophanate

methyl resistant population. Farmers may be able to delay the development of resistance by judicious application of thiophanate methyl avoiding continuous application.

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REFERENCES

Brent, K. J. 1995. Fungicide resistance in crop pathogens: How can it be managed? FRAC monograph No 1, Global crop protection federations, Brussels. 48 pp.

Chung, W., H. Ishii, K. Nishimura, M. Fukaya, K. Yano and Y. Kajitani. 2006. Fungicide sensitivity and phylogenic relationship of anthracnose fungi isolated from various fruit crops in Japan. Plant Disease. 90(4):506-512.

Davidse, L. C. 1986. Benzimidazole fungicides: Mechanism of action and biological impact. Annual Reviews of Phytopathology. 24:43-65.

De Waard, M. A., S. G. Georgopoulos, D. W. Hollomon, H. Ishii, P. N.N. Leroux, Ragsdale and F.J. Schwinn. 1993. Chemical control of plant diseases Problems and prospects. Annual Reviews of Phytopathology. 31:403-421.

Ishii, H., S. Iwamoto, K. Nishimura and M. Fukaya. 1998. Comparative studies on fungicide sensitivity and other characteristics in Colletotrichum isolates from various plant species. Proceedings of the Brighton crop protection conference – Pest and Diseases. November 20-25, 1998, Brighton, UK, pp 529-534.

Kim, Y., J. Y. Min, B. K. Kang, N. V. Bach, W. B. Chor, E. W. Park and H. T. Kim. 2007. Analysis of the less benzimidazole – sensitivity of the isolates of Colletotrichum spp. causing the anthracnose in pepper and strawberry. Plant Pathology. 23(3):187-192.

Kumar, A. S., N. P. Reddy, K. H. Reddy and M. C. Devi. 2007. Evaluation of fungicidal resistance among Colletotrichum gloeosporioides isolates causing mango anthracnose in Agri export zone of Andra Pradesh. Indian Plant Pathology Bulletin. 16:157-160.

LaMondia J.A., 2001. Management of Euonymus anthracnose and fungicide resistance in Colletotrichum gloeosporioides by alternating or mixing fungicides. Journal Environmental Horticulture. 19(1):51-55.

Nalumpang, S., Y. Miyamoto, C. Miyake, Y. Izumi, K. Akitmitsu and P. Kongtragoul. 2010. Point mutations in the beta-tubulin gene conferred carbendazim-resistant phenotypes of Colletotrichum gloeosporioides causing Nam Dok mai mango anthracnose Journal of Agricultural Technology. 6(2): 365-378

Nene, Y. L. and P. N. Thapliyal. 1993. Evaluation of fungicides. In: Fungicides in plant disease control. Pp 526-531. Oxford and IBH Publishing Co., Pvt. Ltd., New Delhi.

Rajapaksha, R. G. A. S., S.P. Edirimanna, G. Weeraratne, D. R. Yapa, G. Abeywickrama, and R. Hemananda. 2001. Management of leaf twister disease of shallot in Kollonna area (IM2 agro-ecological region). Annals of the Sri Lanka Department of Agriculture. 3:201-210.

Rangaswami, G., and A. Mahadeven. 1999. Diseases of crop plants in India. Prentice Hall of India Pvt. Ltd., New Delhi.

Sariah, M. 1989. Detection of benomyl resistance in the anthracnose pathogen, Colletotrichum capsici. Journal of Islamic Academy of Sciences. 2(3):168-171.

Staub, T. 1991. Fungicide resistance: Practical experience with antiresistant stratergies and the role of integrated use. Annual Reviews of Phytopathology. 29:421-442.

Sutton, B.C. 1980. The coelomycetes, fungi imperfect with pycnidia acervuli and stromata. 696 p. CMI, Kew Surrey, England.

Waller, J.M. 1992. Colletotrichum diseases of perennial and other cash crops. In: Colletotrichum: Biology, Pathology and Control. Eds. J.A. Bailey and M J. Jeger. Pp. 167-185. CAB Intl. Wallingford, UK.

Weeraratne, W. A. P. G. 1996 & 1997. Leaf twister disease of onion (*Allium cepa* L.). Tropical Agriculturist. 151:25-33.