Kasetsart J. (Nat. Sci.) 48 : 175 - 182 (2014)

Sexual Reproduction of *Setosphaeria turcica* in Natural Corn Fields in Thailand

Warapon Bunkoed[1, 2, 3], Supot Kasam[1], Patcharavipa Chaijuckam[1], Jeeranan Yhamsoongnern[2] and Sutruedee Prathuangwong[1, 3, 4, *]

ABSTRACT

Setosphaeria turcica, a teleomorph of *Exserohilum turcicum*, is the casual agent of northern corn leaf blight, a major disease of corn in Thailand. It is a heterothallic fungus, with two mating types: mating type A and mating type a. This study was the first to investigate the sexual reproduction of *S. turcica* in Thailand. Pseudothecia (sexual state) were found on heavily infected corn leaves from natural fields, even though until this time, the sexual state has not yet been discovered in the natural world. *S. turcica* isolates collected from nine corn fields were capable of sexual reproduction in culture when opposite mating types were paired, regardless of the origin of the isolates. Pseudothecia, asci and ascospores could be induced on potato dextrose agar and on Sach's agar at 23ºC and 25-30ºC. This study designed mating type-specific primers for both mating types and identified mating type of 225 *S. turcica* isolates. The result revealed near mating type equilibrium in that 104 and 121 isolates were mating type A and mating type a, respectively. Both mating types were present in every field population. The data suggested that sexual reproduction of *S. turcica* may be common in corn fields in Thailand and has caused genetic variation in the fungal pathogen, supported by previous analysis with inter-simple sequence repeat markers. Furthermore, the virulence may be enhanced or new physiological races may be generated through sexual hybridization.

Keywords: *Exserohilum turcicum*, teleomorph, pseudothecia, sexual reproduction

INTRODUCTION

Setosphaeria turcica (Luttr.) K.J. Leonard & Suggs (anamorph: *Exserohilum turcicum* (Pass.) K.J. Leonard & Suggs) is an Ascomycete formerly called *Helminthosporium turcicum* and is the causal agent of northern corn leaf blight (NCLB). NCLB is one of the major diseases of corn throughout the world in temperate climates and in mid-altitude and highland areas of the subtropics and tropics (Renfro and Ullstrup, 1976; Adipala *et al.*, 1993), such as Thailand, particularly where temperatures during the growing season are moderate (15–25 ºC) and there are heavy dew conditions (Levy and Cohen, 1983). This pathogen causes yield losses of up to 50%, especially when the disease occurs early in the season (Perkins and Pederson, 1987; Agrios, 2005). The pathogen reproduces mainly by conidia formed on infected corn leaves during the corn growing season and

1 Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
2 National Corn and Sorghum Research Center, Pak Chong, Nakhon Ratchasima 30320, Thailand.
3 Center for Advanced Studies in Tropical Natural Resources, Kasetsart University, Bangkok 10900, Thailand.
4 Kasetsart University Corn Excellent Center, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: agrsdp@ku.ac.th

Received date : 11/06/13 Accepted date : 12/10/13

overwinters by producing chlamydospores in corn debris (Shurtleff, 1980). *S. turcica* is a heterothallic fungus, having a single-locus system with two mating types: mating type A and mating type a (Nelson, 1959). Previous to the current study, the sexual state had not yet been found in the natural world (Mendes *et al.,* 1987; Gao, 2009). The pathogen may reproduce sexually in the laboratory by producing ascocarp (pseudothecia) on synthetic media containing sterilized plant materials. In the laboratory, ascocarp form when two different mating types (A and a) are grown on barley grain or straw (Luttrell, 1958). In northern China, isolates of *S. turcica* were grouped into three mating types: A, a and Aa. The Aa strains, which were first found in China, were compatible with A and a strains (Yongshan *et al.*, 2007). Random amplification of polymorphic DNA (RAPD) analysis revealed high genotypic diversity among *S. turcica* in Northern China indicating that sexual recombination could produce significant genetic variation in the fungal pathogen (Yongshan *et al.,* 2007). In Thailand, the teleomorph of *S. turcica* in nature has never been reported, but a previous study found that *S. turcica* isolates produced significant genetic variation when assessed using inter simple sequence repeat (ISSR) markers (Bunkoed *et al.,* 2012). The current study searched for sexual structures of *S. turcica* in corn fields in Thailand, identified the mating type of collected *S. turcica* isolates and examined the sexual compatibility between different isolates of *S. turcica* from Thailand on culture media to determine the cause of genetic diversity.

MATERIALS AND METHODS

Teleomorph formation of *Setosphaeria turcica* in nature

Corn leaves with typical northern corn leaf blight symptoms were collected from nine fields where commercially cultivated cultivars were grown, in different agroclimatic zones in Thailand (Table 1). Lesions of infected leaves were sampled and pseudothecia were observed under stereo and light microscopes.

Isolation of *Setosphaeria turcica*

Isolation of *S. turcica* from the infected samples was carried out by picking a conidium from an infected lesion and placing the conidium on water agar (agar 20 g, water 1 L). Incubation was done at room temperature (25–30 ^{o}C) for 72 hr. A hyphal tip from a single conidium was transferred to potato dextrose agar (PDA) as pure culture. The pure cultures were maintained on PDA slants and kept in a refrigerator at 5 ^{o}C for further study.

Table 1 Origin of *S. turcica* isolates collected from nine corn fields in Thailand.

Location	Date	Number of infected leaves
Maejam, Chiang Mai	July 2012	8
Samoung, Chiang Mai	September 2012	16
Tak Fa, Nakhon Sawan	September 2012	13
National Corn and Sorghum Research Center, Nakhon Ratchasima (Suwan Farm)	November 2012	75
Pak Chong, Nakhon Ratchasima	November 2012	17
Phrom Khiri, Nakhon Si Thammarat	November 2012	40
Phop Phra, Tak	January 2013	22
Mae Sot, Tak	January 2013	18
Mae Ramart, Tak	January 2013	16
Total		225

DNA extraction

DNA of each *S. turcica* isolate was extracted by modified cetyl trimethyl ammonium bromide (CTAB) extraction method according to Moller *et al.* (1992). A sample of 30 mg of fungal mycelia was scraped from 10-day-old cultures on PDA, manually ground in 1.5 mL microfuge tubes with a micro pestle in 700 µL of pre-warmed 60 ºC CTAB buffer and incubated at 60 ºC for 60 min. DNA was extracted by adding equal volumes of chloroform:octanol (24:1) and centrifuging at 14,000 rpm for 10 min followed by precipitation by adding 700 µL of cold isopropanol and centrifuging at 14,000 rpm for 10 min. The DNA was washed twice with 70% ethanol, then suspended in 200 µL of Tris ethylene-diamine-tetraacetic acid (TE) buffer and stored at -20 ºC for further use.

Identification of mating type by mating type-specific primers

Sexual reproduction in fungal species is controlled by a mating type gene—mating type A controlled by the *MAT1* gene and mating type a controlled by the *MAT2* gene based on Gao (2009). In the current study, a standard polymerase chain reaction (PCR) protocol was used to amplify the *MAT1* and *MAT2* gene region. Mating type-specific primers MAT1-F 5′-ATGCCACCTCACAAACCTTC-3′ and MAT1–R 5′-TGTGCGCATTAGCAATGTCT-3′ were designed to investigate the *MAT1* gene. Primer MAT2–F 5′-AGCAGTCGTTTCGAGGGTTA-3′ and MAT2–R 5′-CCGTTGTTCCAGTCGTTCTT-3′ were designed to investigate the *MAT2* gene. These primers generated 203 and 199 bp products from A and a strains, respectively. PCR amplification was carried out in a 10 µL reaction mixture, containing 20 ng of template DNA, 1 mM of each primer (BioDesign, Thailand), 100 mM of each dNTP, 2 mM $MgCl_2$ and 0.2 unit of Taq DNA polymerase. The amplification reaction was performed with a program consisting of a predenature at 94 ºC for 3 min, 35 cycles (94 ºC for 1 min, 54 ºC for 1 min and 72 ºC for 2 min) and a final extension at 72 ºC for 4 min. The PCR products were electrophoresed on 1.6% agarose gels and stained with ethidium bromide before visualizing under ultraviolet light.

Teleomorph formation of *Setosphaeria turcica* in the laboratory

Teleomorph formation of *S. turcica* isolates was examined by crossing each pair of six selected isolates in all possible combinations. These six isolates were selected from two locations where pseudothecia were found in natural fields (on Suwan Farm and in a corn field at Pak Chong, Nakhon Ratchasima) and from a location where pseudothecia had not previously been discovered at Samoung, Chiang Mai. A pair of the isolates was placed on opposite sides of a 4-cm-long piece of sterile corn leaf on a dish containing medium. Three replications of each crossing isolate were performed on PDA and Sach's agar (Luttrell, 1958) and incubated at 23 ºC or room temperature (approximately 25–30 ºC) or, for cycles of 16 hour light and 8 hour dark for 3 wk (Ueyama and Tsuda, 1975).

RESULTS

Teleomorph formation of *Setosphaeria turcica* in nature

A search was conducted for teleomorph of *S. turcica* in nine corn fields. Sexual structures were discovered from two fields (Suwam Farm and Pak Chong, Nakhon Ratchasima). Pseudothecia were found on 3 out of 75 infected leaves in the mature stage of susceptible waxy corn on Suwan Farm and on only 1 out of 17 infected leaves of the susceptible sweet corn hybrid at Pak Chong, Nakhon Ratchasima during harvesting.

Pseudothecia were black, globose to ellipsoidal, 40–56 × 12–15 µm with clearly visible setae around the ostiole (Figures 1a and 1b). Asci were cylindrical, clavate, short pedicellate and bitunicate and 1–8 ascospores were observed (Figure 1c).

Figure 1 Perfect state of *S. turcica* from natural corn fields: (a) Pseudothecium on lesion of infected corn leaf (arrow); (b) Psudothecium with septate setae; and (c) Asci consisting of ascospores.

Identification of mating type by mating type-specific primers

The specific primers were designed based on the *MAT1* and *MAT2* genes sequenced by Gao (2009). The *MAT1* gene was cloned from mating type A strains and the *MAT2* gene was cloned from mating type a strains (Gao, 2009). The *MAT1* gene was investigated using primer MAT1-F, and MAT1-R and 203 bp products were generated from mating type A strains, but not from mating type a strains. The *MAT2* gene was investigated using primer MAT2-F, and MAT2-R and 199 bp products were generated from mating type a strains, but not from mating type A strains (Figure 2).

Identification of the mating type of 225 *S. turcica* isolates revealed near mating type equilibrium with 104 and 121 isolates of mating type A and mating type a, respectively. Both mating types occurred in every field population (Table 2).

Teleomorph formation of *Setosphaeria turcica* in laboratory

There were 18 crossing reactions of 36 crossing combinations between 6 selected *S. turcica* isolates that reproduced sexually in the laboratory by producing pseudothecia after 3 wk at 23 ºC or at 25–30 ºC for cycles of 16 hour

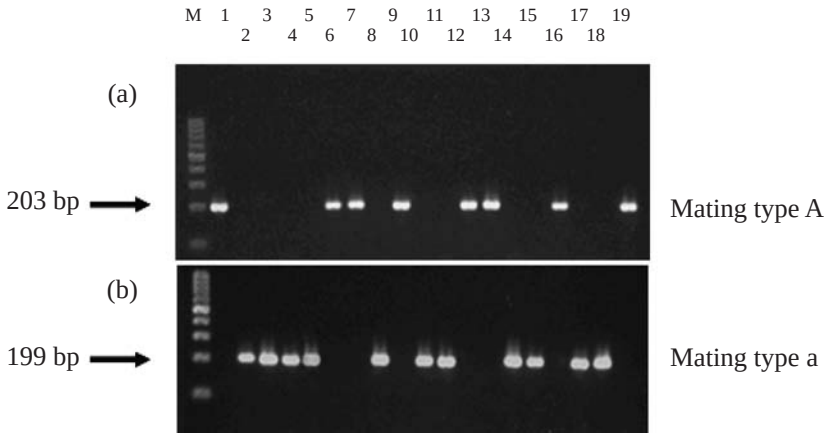

Figure 2 Amplification pattern of *S. turcica* mating types with specific primers of mating type gene, where M is the DNA marker and 1–19 are isolates of *S. turcica* from different fields with specific primers of (a) *MAT1* gene; and (b) *MAT2* gene.

light and 8 hour dark on both PDA and Sach's agar with sterile corn leaf (Table 3). Pseudothecia were observed when opposite mating types were paired regardless of the origin of the isolates. Pseudothecia were produced superficially and on the immersed surface of corn leaf in both media at both temperatures (Figure 3a). However, some of them were embedded in the surface of Sach's agar (Figure 3b) but not on PDA.

Pseudothecia were dark brown to black, globose to ellipsoidal, 250–725 µm in height and 230–450 µm in diameter. Setae exhibited short, stiff, brown, spin-like hairs around the ostiole. Asci were cylindrical, clavate, short pedicellate, bitunicate, with 1–8 ascospores, 175–250 × 24–31 µm (Figure 3c). Ascospores were hyaline, fusoid, 1–6 (mostly 3) septate, straight to slightly curved, constricted at the septa and 30–75 × 10–25 µm (Figure 3d). These characteristics and sizes were consistent with *S. turcica* described by Luttrell (1958). In the crossing examination, 2–44 pseudothecia per plate were found on compatible mating culture.

Table 2 Mating type determination in several isolates of *S. turcica.*

Location	Number of isolates	Mating type	
		A	a
Maejam, Chiang Mai	8	3	5
Samoung , Chiang Mai	16	7	9
Tak Fa, Nakhon Sawan	13	11	2
National Corn and Sorghum Research Center (Suwan Farm), Nakhon Ratchasima	75	48	27
Klang Dong, Pak Chong, Nakhon Ratchasima	17	6	11
Promkiri, Nakhon Si Thammarat	40	5	35
Phop Phra, Tak	22	8	13
Mae Sot, Tak	18	9	10
Mae Ramart, Tak	16	7	9
Total	225	104	121

Table 3 Investigation of teleomorph formation by crossing six selected *S. turcica* isolates in all possible combinations on potato dextrose agar and Sach's agar at 23 °C or 25–30 °C.

Selected isolates		Pseudothecium					
		Mating type A			Mating type a		
		14SW	19KL	15SM	1SW	18KL	4SM
Mating type A	14SW	-	-	-	+	+	+
	19KL	-	-	-	+	+	+
	15SM	-	-	-	+	+	+
Mating type a	1SW	+	+	+	-	-	-
	18KL	+	+	+	-	-	-
	4SM	+	+	+	-	-	-

SW = Selected isolates from Suwan Farm, Nakhon Ratchasima.

KL = Selected isolates from Klang Dong, Pak Chong, Nakhon Ratchasima.

SM = Selected isolates from Samoung, Chiang Mai.

- = Absence of pseudothecium.

+ = Presence of pseudothecium.

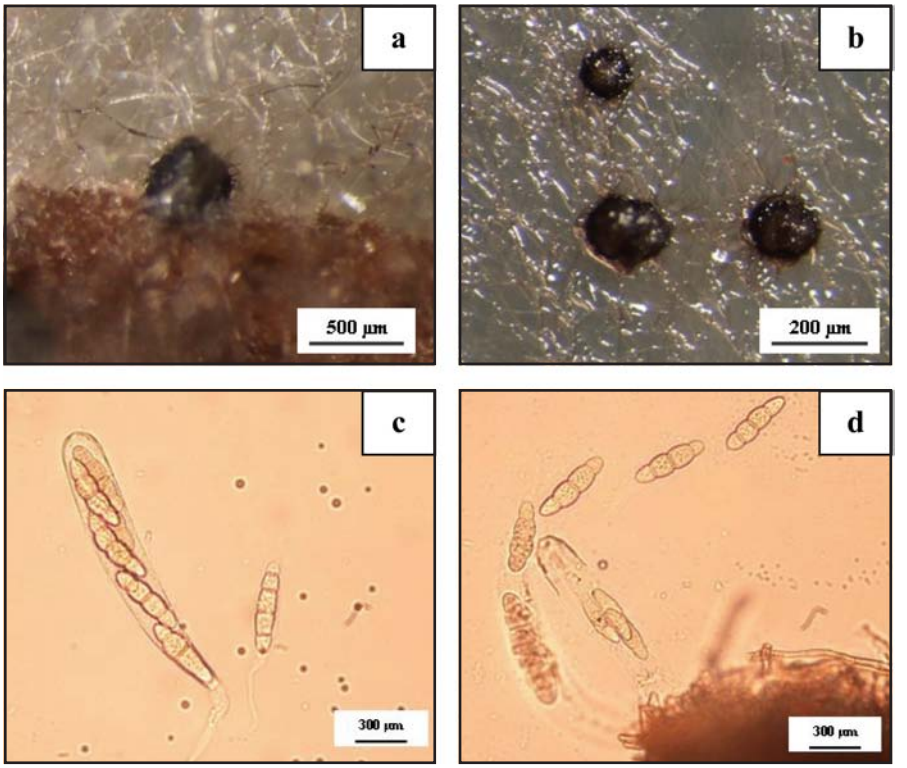

Figure 3 Perfect state of *S. turcica* on Sach's agar with piece of sterile corn leaf: (a) Pseudothecium on corn leaf; (b) Pseudothecia embedded in Sach's agar; (c) Bitunicate ascus (arrowed) consists of Ascospores; and (d) Ascospores (d).

DISCUSSION

The sexual stage of *S. turcica* has never been reported in Thailand. This study represents the first report investigating the teleomorph of *S. turcica* in Thailand. *S. turcica* isolates from corn fields were capable of sexual reproduction in culture when opposite mating types were paired, regardless of the origin of the isolates. Similar results were reported by Luttrell (1958) and Chang and Fan (1986). Sexual structures could be induced on corn leaf in PDA and Sach's agar plates after 3 wk at 23 ºC and 25–30 ºC. Yongshan *et al*. (2007) successfully produced ascocarps of *S. turcica* in Sach's agar with barley culm as the mating stimulator after 4 wk co-incubation of two opposite mating type isolates at 25 ºC in darkness. Morita *et al.* (2012) discovered the development of the perfect stage of *Exserohilum monoceras* on Sach's agar with corn leaf at a constant temperature of 25 ºC with cycles of 16 hr light and 8 hr dark for 3 wk. The Illinois isolates of *S. turcica* produced sexual structures on lactose casein hydrolysate agar (Abadi *et al*., 1993).

S. turcica is a heterothallic fungus, with two mating types: mating type A and mating type a. Mating type-specific primers could identify the mating type of 225 *S. turcica* isolates and revealed that 104 isolates were mating type A and 121 isolates were mating type a. In this study, both mating types were present in every field population. Borchardt *et al.* (1998) reported that tropical populations of *S. turcica* have both mating types in every field population, mostly at equal frequencies They also reported that the sexual stage had not been observed in the field.

While they were uncertain exactly where and when sexual reproduction would occur, they assumed it would be on a maturing or senescent host plant. Data from the current study suggested that sexual recombination occurred in Thailand due to the presence of both mating types and the formation of pseudothecia in two out of nine natural corn fields, even though the sexual state had not yet been discovered in the natural world (Borchardt *et al.,* 1998; Gao, 2009). In natural corn fields, pseudothecia were found on heavily infected leaves at the mature stage of susceptible waxy corn and a susceptible sweet corn hybrid. The pseudothecium from natural fields was smaller than that from laboratory culture and had clearly visible septate setae. Growing susceptible corn continuously may enhance sexual reproduction and pseudothecium formation in natural fields. Borchardt *et al*. (1998), reported that less sexual recombination occurred in temperate climates (Northern China and Europe), as indicated by the strong gametic phase disequilibrium and uneven frequencies of mating types in the pathogen population. The climatic requirements for induction and maturation of fertile pseudothecia may not be fulfilled or may be only seldom fulfilled (Borchardt *et al.,* 1998). In Europe, where *S. turcica* epidemics are often less severe than in the tropics (Smith *et al.,* 1988), pathogen population densities may be too low to allow frequent contact of sexually compatible isolates. However, when the severity of epidemics increases, the distribution of mating types within fields may become more random and enhance the chance for sexual reproduction (Borchardt *et al.,* 1998). At least in years with favorable conditions (temperature, moisture and appropriate substrates from host tissue), sexual recombination may also occur in temperate zones (Borchardt *et al.,* 1998).

Sexual recombination causes an increase in genotypic diversity (Stoddart and Taylor, 1988). In a previous study, *S. turcica* isolates in Thailand were found to produce significant genetic variation when assessed by using inter simple sequence repeat (ISSR) markers (Bunkoed *et al.,* 2012). Therefore, sexual recombination is possibly one of the major sources for genetic variation in *S. turcica* in Thailand. Tropical populations based on 264 *S. turcica* isolates (from Kenya, Mexico and southern China) were analyzed with 70 RAPD markers and their mating types were determined. The results showed extremely high genotypic diversity, no or only weak gametic phase disequilibrium and an even distribution of the two mating types, indicating frequent sexual recombination (Borchardt *et al.,* 1998). Sexual hybridization is one of the main paths which lead to fungal variations and enhance the mutation rate (Gao, 2009). The virulence may be enhanced or new physiological races may be generated through sexual hybridization (Gao, 2009; Ni *et al.,* 2011). The results indicated that *S. turcica* may have the potential to cause severe epidemics in major corn growing locations in Thailand. Such information may strategically support resistance breeding programs.

CONCLUSION

The perfect stage of *S. turcica* could be induced on PDA and Sach's agar with sterile corn leaf at 23 ^{o}C and 25–30 ^{o}C. Pseudothecia were observed on lesions of infected corn leaves from natural corn fields. Mating type-specific primers could identify the mating type of 225 *S. turcica* isolates and revealed that 104 isolates were mating type A and 121 isolates were mating type a. Both mating types were present in every field population.

ACKNOWLEDGEMENT

The authors express their thanks to the National Corn and Sorghum Research Center. This research was supported by the Center for Advanced Studies in Tropical Natural Resources, Kasetsart University.

LITERATURE CITED

Abadi, R., R.S. Levy and Y. Levy. 1993. Mating type of *Exserohilum turcicum* in Israel. **Phytoparasitica** 21(4): 315–320.

Adipala, E., P.E. Lipps and L.V. Madden. 1993. Occurrence of *Exserohilum turcicum* on maize in Uganda. **Plant Dis.** 77: 202–205.

Agrios, G.N. 2005. **Plant Pathology.** 5th ed. Elsevier Academic Press. Burlington, USA. 922 pp.

Borchardt, D.S., H.G. Welz and H.H. Geiger. 1998. Genetic structure of *Setosphaeria turcica* populations in tropical and temperate climates. **Phytopathology** 88: 322–329.

Bunkoed, W., S. Kasam, P. Chaijuckam, W. Yenpet, J. Yhamsoongnern and S. Prathuangwong. 2012. An ISSR marker characterizes molecular variability of *Exserohilum turcicum* isolates on maize. *In* **The International Conference on Tropical and Sub-Tropical Plant Diseases**, 7–10 February 2012. The Empress Hotel, Chiang Mai, Thailand.

Chang, H.S. and K.C. Fan. 1986. Comparative studies on some biology and pathology of corn and broom corn isolates of *Exserohilum turcicum* (Pass) Leonard and Suggs. **Bot. Bull. Acad. Sin. (Taipei)** 27: 209–218.

Gao, R. 2009. **Cloning of MAT Genes and Fertility Analysis of the Bisexual Strain of *Setosphaeria turcica*.** Masters thesis, Baoding, P.R. China. 42 pp.

Levy, Y. and Y. Cohen. 1983. Biotic and environmental factors affecting infection of sweet corn with *Exserohilum turcicum*. **Phytopathology** 73: 722–725.

Luttrell, E.S. 1958. The perfect stage of *Helminthosporium turcicum*. **Phytopathology** 48: 281–287.

Mendes, M.C., E.H. Sobrinho and R.T. Guerreiro. 1987. Sexual compatibility in *Trichometasphaeria turcica* Lutt. **Brazil. J. Genetic** 4: 647–653.

Moller, E.M., G. Bahnweg, H. Sandermann and H.H. Geiger. 1992. A simple and efficient protocol for isolation of high molecular weight protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissue. **Nucleic Acid Res.** 20: 6115–6116.

Morita, A., Y. Saitoh, K. Izumitsu and C. Tanaka. 2012. Teleomorph formation of *Setosphaeria monoceras*, a perfect state of *Exserohilum monoceras*, by Japanese isolates. **Mycoscience** 53(2): 144–146.

Nelson R.R. 1959. A major gene locus for compatibility in *Trichometasphaeria turcica*. **Phytopathology** 49: 159–161.

Ni, M., M. Feretzaki, S. Sun, X. Wang and J. Heitman. 2011. Sex in Fungi. **Annu. Rev. Genet.** 45: 405–430.

Perkins, J.M. and W.L. Pederson. 1987. Disease development and yield losses associated with northern corn leaf blight on corn. **Plant Dis.** 71: 940–943.

Renfro, B.L. and A.J. Ullstrup. 1976. A comparison of maize diseases in temperate and in tropical environments. **Proc. Natl. Acad. Sci.** 22: 491–498.

Shurtleff, M.C. 1980. **Compendium of Corn Diseases.** American Phytopathological Society. St. Paul, MN, USA. 105 pp.

Smith, I.M., J. Dunez, R.A. Lelliott, D.H. Phillips and S.A. Archer. 1988. **European Handbook of Plant Diseases.** Blackwell Scientific. Oxford, UK. 583 pp.

Stoddart, J.A. and J.F. Taylor, J.F. 1988. Genotypic diversity: Estimation and prediction in samples. **Genetics** 118: 705–711.

Ueyama, A. and M. Tsuda.1975. Formation in culture of *Cochliobolus miyabeanus*, the perfect state of *Helminthosporium oryzae*. **Ann. Phytopathol. Soc. Jpn.** 41: 434–440.

Yongshan, F., M. Jifang and G. Xiumei. 2007. Distribution of mating types and genetic diversity induced by sexual recombination in *Setosphaeria turcica* in Northern China. **Frontiers of Agriculture in China** 1: 368–376.