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Research Article

Excised Leaf Method for High Volume Evaluation of Sorghum Germplasm for Resistance Against *Colletotrichum sublineolum*

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Abstract

Foliar phase of anthracnose, caused by *Colletotrichum sublineolum* is the most important leaf disease of sorghum. Due to the hyper-variable nature of the fungus, continuous evaluation of sorghum germplasm to identify new sources of resistance is imperative. Field and greenhouse evaluations for anthracnose resistance, especially with large numbers of sorghum lines/accessions can be expensive, time consuming and require large spaces and labor. In this study, 16 sorghum lines were evaluated by putting a drop of the mixture of *C. sublineolum* isolates suspension on each side of the midrib of adaxial excised leaves plated on half-strength potato dextrose agar medium and concurrently as whole plants inoculated with a mixture of *C. sublineolum* isolates-colonized grain and conidial suspension in the greenhouse. Each line exhibited the same reaction when challenged with *C. sublineolum* either using the excised leaf assay or screened in the greenhouse, indicating that the excised leaf assay is as effective in identifying susceptibility or resistance to the anthracnose pathogen. In both screening methods, SC748 was the only resistant line. The excised leaf assay was completed in 4 days while the greenhouse evaluation was arrested 44 days post-inoculation. Thus, the excised leaf method could offer several advantages in screening sorghum for anthracnose resistance such as reducing the time for conducting the experiment, labor, space and increasing the number of isolates that can be tested within a short period.

Key words: *Sorghum bicolor*, *Colletotrichum sublineolum*, anthracnose, screening method, excised leaf assay

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sorghum anthracnose, caused by *Colletotrichum sublineolum* P. Henn., in Kabat and Bubák (syn. *C. graminicola* (Ces.) G. W. Wilson), occurs in most sorghum-growing regions worldwide, with the foliar phase of the disease causing the most damage (Ali and Warren, 1987; Pastor-Corrales, 1980; Sherriff *et al*., 1995; Thakur and Mathur, 2000). Foliar infection can occur at any stage of plant development but symptoms are generally observed 40 days after seedling emergence, initially appearing as small circular to elliptical spots or elongated lesions and subsequently, as the fungus sporulates, fruiting bodies (acervuli) appear as black spots in the center of the lesions (Thakur and Mathur, 2000). Estimating grain yield losses due to foliar anthracnose infection is difficult (Ngugi *et al*., 2000) but losses as high as 50% have been reported in susceptible cultivars (Harris *et al*., 1964; Powell *et al*., 1977; Thomas *et al*., 1996). The occurrence of different pathotypes and levels of pathogenicity within the pathogen population require continual screening to identify new sources of resistance (Ali and Warren, 1987; Cardwell *et al*., 1989; Pande *et al*., 1991; Casela *et al*., 1992; Prom *et al*., 2012b). Reliable and efficient inoculation techniques for mass evaluation of sorghum germplasm for anthracnose resistance in the field and greenhouse have been developed (Pande *et al*., 1994; Thakur and Mathur, 2007; Prom *et al*., 2009). However, field and greenhouse evaluations for anthracnose resistance, especially when dealing with large numbers of sorghum lines/accessions are costly, time consuming and require large inputs of space and labor. The use of excised leaf assay to either evaluate lines/cultivars for resistance or determine the efficacy of fungicides and plant extracts to a number of plant pathogens have been documented (Seema *et al*., 2011; Truong *et al*., 2012; Metz *et al*., 2012; Ammar *et al*., 2013; Rahman *et al*., 2013; Nowakowska *et al*., 2014). The use of excised leaf to determine the efficacy of fungicides and potassium phosphonate against *Pestalotia palmarum* and *Phytophthora capsici*, respectively, was shown to be highly reliable (Truong *et al*., 2012; Rahman *et al*., 2013). Metz *et al*. (2012) evaluated a number of St. Augustine grass genotypes against *Magnaporthe oryzae* using detached leaf assay and noted that the results correlated highly with those obtained from the field and growth chamber experiments. The use of excised leaf assay was shown to be rapid and cost effective in evaluating *Citrus* spp., against the citrus huanglongbing disease pathogen (Ammar *et al*., 2013). Similarly, the evaluation of tomato genotypes for resistance to late blight using detached leaf assay was shown to correlate highly with results obtained from field tests (Nowakowska *et al*., 2014). In this communication, reported the use of an excised leaf assay to rapidly assess sorghum germplasm for resistance against *C. sublineolum* in the laboratory and to determine concurrently the response of the same lines to anthracnose infection in the greenhouse.

MATERIALS AND METHODS

Seeds of 16 sorghum lines, including BTx623, PI609251 and TAM 428, used as susceptible checks and SC 748-5,used as a resistant check, were grown in the greenhouse in 3-gallon Poly-Tainer Cans (10"x91/2"x85/8") (Poly-Tainer, Inc., Simi Valley, CA) containing Metro Mix 200 potting soil (Sun Gro Horticulture, Agawam, MA) and Osmocote Classic fertilizer 17-7-12 (O.M. Scott and Sons Company) (1:50 w/w fertilizer:soil). Each line was replicated three times in a complete randomized design, with eight seeds per replicate. In addition, all plants were fertilized on a bi-weekly basis with Peter Excel Multi-purpose 15-5-15 (O.M. Scott and Sons Company). At the 8-leaf stage, the sixth leaf was cut into pieces and placed in individually marked plastic bags and transported to the laboratory. The excised leaves were plated in half-strength potato dextrose agar (½ PDA) and inoculated with a mixture of *C. sublineolum* isolates within 30 min.

Laboratory protocol: Adaxial leaf pieces of each line were plated (2-3 pieces per plate, replicated three times) on half-strength potato dextrose agar medium (½ PDA). Eight *C. sublineolum* isolates (FSP 2, FSP 5, FSP 7, FSP 35, FSP 36, FSP 44, FSP 50 and FSP 53) were grown separately in petri plates containing ½ PDA and incubated at 28°C for 10 days in the dark. Conidia for the different isolates were obtained by adding 10 mL sterilized water to each plate and scraping the agar surface with a rubber spatula. The conidia suspensions were filtered through four layers of sterile cheese cloth into a single beaker and diluted with sterile water to final concentration of 1×10^6 conidia per milliliter. Tween 20 was added to the suspension at a rate of 0.5 mL L^{-1}. A drop of the mixture of the *C. sublineolum* isolates suspension was placed at each side of the excised leaves and the plates were incubated at 28°C for 4 days in the dark (Fig. 1a). The excised leaves were examined and photographed using the 10X objective of a Leica DMLB compound microscope. Plates were scored as either susceptible [positive (+)] i.e., the presence of masses of pink or dark brown conidia oozing with long setae from acervuli (Fig. 1a-b) or resistant [negative (-)] i.e., absence of acervuli (Fig. 2a-b).

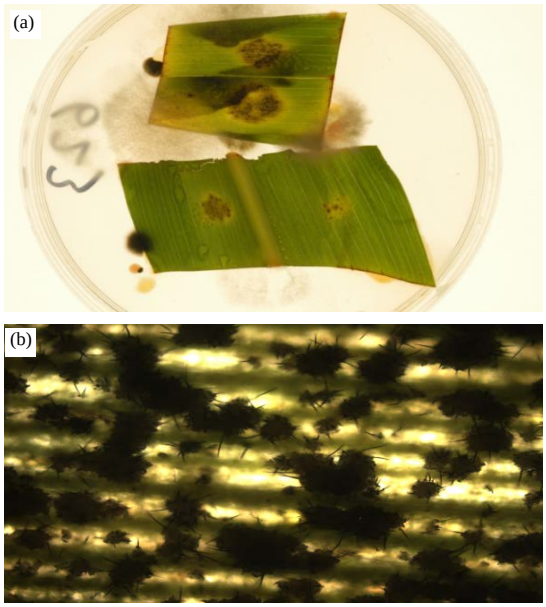

Fig. 1(a-b): (a) Excised sorghum leaf plated on half-strength potato dextrose agar and inoculated by dropping a mixture of *Colletotrichum sublineolum* isolates suspension on each side of the mid-rib. Formation of acervuli 4 d post-inoculation can be seen on the spot where the drop was placed and (b) Presence of masses of pink or dark brown conidia oozing with long setae from acervuli

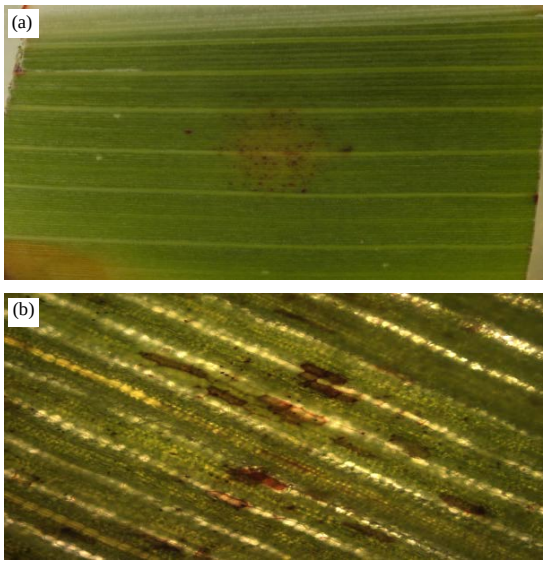

Fig. 2(a-b): (a) Hypersensitive reaction where the drop of *Colletotrichum sublineolum* suspension was placed-no acervuli formation and (b) Necrotic lesions-no development of *Colletotrichum sublineolum*

Fig. 3: Greenhouse grown plants (SC748) inoculated with *Colletotrichum sublineolum*. Plants showing resistant reaction i.e., no symptoms or chlorotic flecks on some of the leaves and on other leaves hypersensitive reaction (small reddening or red spots) on inoculated leaves but no acervuli formation and no lesion development

Greenhouse protocol: Plants that had leaves excised for the *in vitro* assay were concurrently evaluated for disease reaction in the greenhouse using methods previously described by Prom *et al.* (2009). Briefly, at the 8 leaf stage, 10 seeds of the mixture of *C. sublineolum*-isolates-colonized sorghum were placed into the leaf whorl of each plant (15 plants per line). Plants were also sprayed with a conidia suspension of a mixture of the *C. sublineolum* isolates. In order to provide an environment conducive to infection and disease development, plants were misted 30 min after the spray inoculation for 30 sec at 45 min intervals for 8 h per day for 30 day. The plants were rated 30 day post-inoculation and weekly thereafter for two weeks. The ratings were based on a scale of 1-5 (Prom *et al.*, 2009): 1 = No symptoms or chlorotic flecks on leaves, 2 = Hypersensitive reaction (reddening or red spots) on inoculated leaves but no acervuli formation and no lesion development on other leaves, 3 = Lesions on inoculated and bottom leaves with acervuli in the center, 4 = Necrotic lesions with acervuli on inoculated leaves and infection spreading to bottom and middle leaves and 5 = Most leaves dead due to infection with infection on the flag leaf containing abundant acervuli. Lines were categorized into two reaction classes, resistant if plants in the cans were rated as 1 or 2 (Fig. 3) and susceptible if rated as 3, 4, or 5 (Fig. 4).

Fig. 4: Greenhouse grown plants (TAM428) inoculated with *Colletotrichum sublineolum*. Plants showing susceptible reaction i.e., lesions on inoculated and bottom leaves with acervuli in the center, necrotic lesions with acervuli on inoculated leaves and infection spreading to middle and upper leaves

Table 1: Reaction of sorghum lines for resistance against *Colletotrichum sublineolum in vitro* and *in vivo*[1]

	Reactions		
Lines	Leaf disk[2]	Greenhouse[3]	
NECS197	+	(5)*	+
NECS239	+	(5)	+
RTx 430	+	(3)	+
NECS138	+	(5)	+
PI609251	+	(5)	+
SC 748-5	-	(2)	-
BTx 623	+	(5)	+
NECS191	+	(4)	+
NECS248	+	(5)	+
NECS116	+	(4)	+
NECS54-2	+	(4)	+
TAM 428	+	(4)	+
NECS601	+	(5)	+
NECS119	+	(4)	+
NECS213	+	(5)	+
NECS22-1	+	(5)	+

[1]Sixteen lines were evaluated for resistance against *C. sublineolum*, causal agent of sorghum anthracnose using an excised leaf option (*in vitro*) in the laboratory and concurrently in the greenhouse (*in vivo*, potted plants). In the laboratory or greenhouse, the scoring was the same i.e., the presence of acervuli determines the reaction class (+ = susceptible and - = resistant), [2]Plates were scored 4 days after inoculation as either susceptible [positive (+)] i.e., the presence of masses of pink or dark brown conidia oozing with long setae from acervuli (Fig. 1a-b) or resistant [negative (-)] i.e., absence of acervuli (Fig. 2a-b), [3]Greenhouse plants were rated using a scale of 1-5 as described by Prom *et al.* (2009) where 1 = no symptoms or chlorotic flecks on leaves; 2 = hypersensitive reaction (reddening or red spots) on inoculated leaves but no acervuli formation and no lesion development on other leaves; 3 = lesions on inoculated and bottom leaves with acervuli in the center; 4 = necrotic lesions with acervuli on inoculated leaves and infection spreading to bottom and middle leaves and 5 = most leaves dead due to infection with infection on the flag leaf containing abundant acervuli. *( ) = mean score for the line. Lines were categorized into two reaction classes, resistant (-) if plants in the cans were rated as 1 or 2 and susceptible (+) if rated as 3, 4, or 5

RESULTS AND DISCUSSION

In vitro screening using leaf disk, leafed single-node cuttings, detached leaves and callus cultures to determine resistance to a number of plant diseases has been previously conducted by a number of researchers (Pandey *et al*., 2011; Ostry, 1997; Pratt, 1996; Liu *et al*., 2003; Khan and Hsiang, 2003; Li *et al*., 2007). In this study, 16 sorghum lines were concurrently screened for resistance against *C. sublineolum* following this newly designed *in vitro*, excised leaf assay and the established, *in vivo* greenhouse protocol (Prom *et al*., 2009). The results of the excised leaf assay revealed that all the lines, except for SC748, were susceptible when inoculated with a mixture of *C. sublineolum* isolates (Table 1). Figure 1 and 2 showed physical proof of the susceptible and resistance responses from the excised leaf assay. Similarly, the results of the greenhouse evaluation showed that SC748 was resistant against the anthracnose pathogen while all the other lines were susceptible (Table 1). The SC748 had been planted in several locations in the US and abroad; as well as, tested against several pathotypes of *C. sublineolum* and in all cases, this line is consistently resistant to anthracnose (Mehta *et al*., 2005; Prom *et al*., 2012a, b). The consistency of resistance or susceptibility when challenged with *C. sublineolum* using excised leaf assay or evaluated using whole plants in the greenhouse (Table 1) suggests that this excised leaf protocol is effective in identifying resistance and susceptibility in sorghum germplasm to anthracnose. While the excised leaf assay required 4 days to complete, the greenhouse evaluation was completed 44 days post-inoculation. However, the leaf assay cannot determine relative disease severity (as percent leaf area infected), whereas, in the greenhouse evaluation the percent leaf area infected in the susceptible lines ranged from 75% (PI609251) to 10% (Tx430) (data not shown).

There are many examples of leaf assays or similar *in vitro* approaches used to evaluate host plant resistance to diseases. The use of excised leaf tissues of different alfalfa genotypes for resistance against *S. trifoliorum* was shown to be a reliable and efficient method (Pratt, 1996). Liu *et al*. (2003) found that

the use of leafed single-node cuttings was effective in separating grapevine (*Vitis* sp.) genotypes for resistance to downy mildew. Li *et al*. (2007) also evaluated 15 cultivars and their hybrids against *Erysiphe pulchra* and observed the leaf disk assay technique as an effective protocol to screen the powdery mildew resistance in dogwood (*Cornus* sp.). Pandey *et al*. (2011) evaluated F_1 progeny of mango (*Mangifera* sp.) hybrids *in vitro* using mango leaves and noted none of the genotype showed immunity to *C. gloesporioides*. Furthermore, Ostry (1997) noted that screening poplar (*Populus* sp.) for resistance against a number of bacterial and fungal pathogens using *in vitro* assays such as leaf disk has certain advantages. These advantages included short duration for the test, reduced cost, ability to set the environmental conditions, ability to screen a large number of genotypes and the use of a single cell or a few number of cells and expose them to the pathogen or host-specific toxin (Ostry, 1997). Ostry (1997) also noted that *in vitro* assays have some disadvantages because host resistance response is complex and involves a series of biochemical activities by the host, pathogen and environmental factors that may be absent during *in vitro* tests.

In conclusion, this excised leaf assay can provide a rapid, cost effective and practical means for distinguishing resistance or susceptibility of sorghum germplasm to anthracnose, especially with a large number of sorghum germplasm. The excised leaf assay could also be used to delimit the different pathotypes of the anthracnose pathogen because the appearance or absence of acervuli determines the reaction type for this pathosystem. This method could offer savings in time and resources required during experiments such as less space needed in greenhouse and conducting multiple studies indoors in winter months, when field spaces are unavailable. The ability to generate numerous leaf pieces per plant and assay each in isolation, *in vitro*, increases the number of isolates that can be tested in a short time period. In 2012, 18 sorghum lines were used as differentials to delimit the pathotypes of *C. sublineolum* (Prom *et al*., 2012b). Using the current method, a single plant of each sorghum differential could be used to determine the virulence pattern of a number of isolates. Furthermore, results can be obtained within 4 days using the excised leaf assay and if confirmation is needed the resistant lines obtained from the leaf assay could be evaluated in replicated trials in the field or greenhouse. Screening vast quantities of the exotic sorghum germplasm is feasible with this faster protocol and avoids the time, cost and challenges associated with field trials.

REFERENCES

Ali, M.E.K. and H.L. Warren, 1987. Physiological races of *Colletotrichum graminicola* on sorghum. Plant Dis., 71: 402-404.

Ammar, E.D., A.J. Walter and D.G. Hall, 2013. New excised-leaf assay method to test inoculativity of Asian Citrus Psyllid (Hemiptera: Psyllidae) with *Candidatus* liberibacter asiaticus associated with citrus huanglongbing disease. J. Econ. Entomol., 106: 25-35.

Cardwell, K.F., P.R. Hepperly and R.A. Frederiksen, 1989. Pathotypes of *Colletotrichum graminicola* and seed transmission of sorghum anthracnose. Plant Dis., 73: 255-257.

Casela, C.R., A.S. Ferreira and R.E. Schaffert, 1992. Physiological Races of *Colletotrichum graminicola* in Brazil. In: Sorghum and Millets Diseases: A Second World Review, De Milliano, W.A.J., R.A. Frederiksen and G.D. Bengston (Eds.). International Crops Research Institute for the Semi-Arid Tropics, India, pp: 209-212.

Harris, H.B., B.J. Johnson, J.W. Dobson Jr. and E.S. Luttrell, 1964. Evaluation of anthracnose on grain sorghum. Crop Sci., 4: 460-462.

Khan, A. and T. Hsiang, 2003. The infection process of *Colletotrichum graminicola* and relative aggressiveness on four turfgrass species. Can. J. Microbiol., 49: 433-442.

Li, Y.H., M.T. Windham, R.N. Trigiano, D.C. Fare, J.M. Spiers and W.E. Copes, 2007. Evaluation for resistance to powdery mildew in *Cornus* species and hybrids using a leaf disk assay. J. Environ. Hortcult., 25: 131-133.

Liu, S.M., S.R. Sykes and P.R. Clingeleffer, 2003. A method using leafed single-node cuttings to evaluate downy mildew resistance in grapevine. Vitis, 42: 173-180.

Mehta, P.J., C.C. Wiltse, W.L. Rooney, S.D. Collins and R.A. Frederiksen *et al*., 2005. Classification and inheritance of genetic resistance to anthracnose in sorghum. Field Crops Res., 93: 1-9.

Metz, S.P., A. Chandra, A.D. Genovesi and C.F. Campos, 2012. Comparison of screening methods to evaluate the response of St. Augustine grass to *Magnaporthe oryzae*. Plant Pathol., 61: 255-261.

Ngugi, H.K., A.M. Julian, S.B. King and B.J. Peacocke, 2000. Epidemiology of sorghum anthracnose (*Colletotrichum sublineolum*) and leaf blight (*Exserohilum turcicum*) in Kenya. Plant Pathol., 49: 129-140.

Nowakowska, M., M. Nowicki, U. Klosinska, R. Maciorowski and E.U. Kozik, 2014. Appraisal of artificial screening techniques of tomato to accurately reflect field performance of the late blight resistance. PloS ONE, Vol. 9. 10.1371/journal.pone.0109328

Ostry, M., 1997. *In vitro* Screening and Selection for Disease Resistance. In: Micropropagation, Genetic Engineering and Molecular Biology of *Populus*, Klopfenstein, N.B., Y.W. Chun, M.S. Kim and M.A. Ahuja (Eds.). RMRS Publication, Logan, UT., USA., pp: 155-160.

Pande, S., L.K. Mughogho, R. Bandyopadhyay and R.I. Karunakar, 1991. Variation in pathogenicity and cultural characteristics of sorghum isolates of *Colletotrichum graminicola* in India. Plant Dis., 75: 778-783.

Pande, S., R.P. Thakur, R.I. Karunakar, R. Bandyopadhyay and B.V.S. Reddy, 1994. Development of screening methods and identification of stable resistance to anthracnose in sorghum. Field Crops Res., 38: 157-166.

Pandey, A., B.K. Pandey, M. Muthukumar, S. Rajan and L.P. Yadava, 2011. *In vitro* screening for resistance to anthracnose (*Colletotrichum gloeosporioides*) in different mango (*Mangifera indica*) hybrids. Tunisian J. Plant Prot., 6: 99-108.

Pastor-Corrales, M.A., 1980. Variation in pathogenicity of *Colletotrichum graminicola* (Cesati) Wilson and in symptom expression of anthracnose of *Sorghum bicolor* (L.) Moench. Ph.D. Thesis, Texas A and M University, College Station, Texas.

Powell, P., M. Ellis, M. Alaeda and A.M. Sotomayer, 1977. Effect of natural anthracnose epiphytotic on yield, grain quality, seed health and seed borne fungi in *Sorghum bicolor*. Sorghum Newslett., 20: 77-78.

Pratt, R.G., 1996. Screening for resistance to *Sclerotinia trifoliorum* in alfalfa by inoculation of excised leaf tissue. Phytopathology, 86: 923-928.

Prom, L.K., R. Perumal, J.E. Erpelding, T. Isakeit, N. Montes-Garcia and C.W. Magill, 2009. A pictorial technique for mass screening of sorghum germplasm for anthracnose (*Colletotrichum sublineolum*) resistance. Open Agric. J., 3: 20-25.

Prom, L.K., J. Erpelding, R. Perumal, T. Isakeit and H. Cuevas, 2012a. Response of sorghum accessions from four African countries against *Colletotrichum sublineolum*, causal agent of sorghum anthracnose. Am. J. Plant Sci., 3: 125-129.

Prom, L.K., R. Perumal, S.R. Erattaimuthu, C.R. Little and E.G. No *et al*., 2012b. Genetic diversity and pathotype determination of *Colletotrichum sublineolum* isolates causing anthracnose in sorghum. Eur. J. Plant Pathol., 133: 671-685.

Rahman, S., S.K. Adhikary, S. Sultana, S. Yesmin and N. Jahan, 2013. *In vitro* evaluation of some selected fungicides against *Pestalotia palmarum* (Cooke.) causal agent of grey leaf spot of coconut. Plant Pathol. Microbiol., Vol. 4.

Seema, M., S.S. Sreenivas, N.D. Rekha and N.S. Devaki, 2011. *In vitro* studies of some plant extracts against *Rhizoctonia solani* Kuhn infecting FCV tobacco in Karnataka light soil, Karnataka, India. J. Agric. Technol., 7: 1321-1329.

Sherriff, C., M.J. Whelan, G.M. Arnold and J.A. Bailey, 1995. rDNA sequence analysis confirms the distinction between *Colletotrichum graminicola* and *C. sublineolum*. Mycol. Res., 99: 475-478.

Thakur, R.P. and K. Mathur, 2000. Anthracnose. In: Compendium of Sorghum Diseases, Frederiksen, R.A. and G.N. Odvody (Eds.). The American Phytopathological Society, St. Paul, MN., USA., pp: 10-12.

Thakur, R.P. and K. Mathur, 2007. Anthracnose. In: Screening Techniques for Sorghum Diseases, Thakur, R.P., B.V.S. Reddy and K. Mathur (Eds.). ICRISAT Publication, Pantacheru, India, ISBN-13: 9789290665045, pp: 15-23.

Thomas, M.D., I. Sissoko and M. Sacko, 1996. Development of leaf anthracnose and its effect on yield and grain weight of sorghum in West Africa. Plant Dis., 80: 151-153.

Truong, N.V., L.W. Burgess and E.C.Y. Liew, 2012. Greenhouse and field evaluations of potassium phosphonate: The control of Phytophthora foot rot of black pepper in Vietnam. Arch. Phytopathol. Plant Protect., 45: 724-739.