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Studies on Soybean pathogens in the Southern Guinea Savanna Zone of Nigeria.

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1 SUMMARY

Pathogens of soybean in the Guinea Savanna Zone of Nigeria were isolated from plants and seed sources and identified. Pathogenicity tests using the blotter method were carried out to determine the virulence of the organisms isolated on seeds, seedlings and leaves of soybean. This would help in the effective protection of the crop in the zone and increase yield, seed quality, and storability. Results showed that eight organisms namely *Aspergillus flavus*, *A. niger, A. tamarii, and Botryodiplodia theobromae* ,were predominantly affecting soybean seeds post harvest while *Phomopsis sojae, Fusarium spp, Cercospora kikuchi* and *Cercospora sojina* were predominantly, affecting soybeans plants in the field. These organisms caused damages like staining of seeds in storage. However, contrary to previous reports, *Aspergillus* spp were found to improve seed viability. Seeds treated with *A. flavus*, *A. niger,* and *A. tamarii* germinated more than the control by 35%, 17.5% and 22.5% respectively. *A. flavus* was found to have protected the seeds up to the 10th month in storage. *A. flavus* therefore has the potential for biological control of other pathogenic organisms of soybean in the zone. Among the organisms isolated, *Phomopsis sojae* was found to be the most virulent organism affecting both plants and seeds of soybean in the zone. Farmers in the zone should take measures that will control these organisms especially *P. sojae* in the field and storage so as to avert losses of soybean in the zone.

2 INTRODUCTION

In many countries, soybean is valued as a productive and adaptable crop which fits well into the cropping patterns under varying agro-climatic conditions (IITA, 2002). The very rapid rise of the soybean among world commercial crops is largely based on continual improvement in cultivars and production technology, and development of other infrastructure essential for commercialisation of soybean (Delouche, 1974).

In tropical countries, the potential for growing soybean has been clearly established; In Nigeria, soybean production has greatly increased especially in Benue State, the largest producer in the Southern Guinea Savanna Zone of the Country (IITA, 1986). With the introduction of high yielding varieties and increased knowledge of soybean culture, coupled with the awareness of its industrial potentials and its role in human and animal nutrition, e.g. vegetable oil production, soybean production in the zone is likely to increase remarkably in the nearest future Okoro (2004). However, as the production of the crop is increasing, soybean diseases have also increased in number and severity, causing yield losses to farmers in the area Okoro (2004). One of the many problems encountered in the

production of soybeans in the humid tropical environments of West Africa, is the wide fluctuation in the germination and emergence of soybean seeds in the field from year to year, such that the target plant population densities cannot be met (IITA, 1974; Okoro and Nwankiti, 2003). The causes of these problems include poor quality seeds (Okoro *et al.,* 2004), poor storage conditions (Delouche, 1981), increased incidence of seed borne fungal (Phomopsis) infection, and sowing and harvesting when environmental conditions are unfavourable. Infections caused by seed-borne pathogens are complicated. Often there is insufficient information on the disease causing organisms. Uncontrolled pathogens results in massive attack of seeds in storage which severely affects their viability and market value (Sinclair and Backman, 1989). The infected seeds are also sources of inoculum in the field when they are planted.

This trial was undertaken to identify the disease causing organisms of soybean in the Southern Guinea Savanna Zone of Nigeria..

3 MATERIALS AND METHODS

3.1 Isolation of seed borne organisms: Four hundred soybean seeds from each of 12 varieties obtained from IITA (Table 1) were surface disinfected for 2 minutes in 3% sodium hypochlorite and rinsed twice with sterile distilled water. Ten seeds per variety were plated per Petri-dish, using the blotter method. The blotter method involves plating of seeds on moistened filter papers and incubating for 7 days at room temperature (28^{o}C) under 12 hours of alternating cycles of light and darkness. At the end of the incubation period, each seed was examined thoroughly under stereomicroscope for the growth of fungi (Mathur *et al;* 1989). The organisms isolated were identified after purification by sub culturing on potato dextrose agar for *Aspergillus flavus, A. niger, A. tamarii, Fusarium sp, Botryodiplodia theobromae, Phomopsis spp,* while V – 8 juice agar was used for *Cercospora sojina* and *C. kikuchi*. Cultures were then identified based on growth patterns, morphological structures of each organism, recommended keys and texts (ISTA, 1981; Kulwant *et al*., 1991; Barnett and Barry, 1987). Each variety was replicated four times (100 seeds/replicate) and arranged in completely randomised design. The experiment was repeated three times. Data collected included organisms isolated, frequency of isolation, germination percentage of seeds and percentage seed infection.

3.2 Isolation of organisms from infected leaves: Diseased soybean leaves (samsoy 2 variety) infected with *Cercospora sojina* frogeye leaf spot and *Phomopsis* leaf spot diseases were harvested in the field and placed in envelopes and carried immediately to the laboratory, where small sections (1-3cm^2) from the margin of lesions on infected leaf were cut, surface sterilised in 3% sodium hypochlorite solution for 1 minute and rinsed in sterile distilled water. Four pieces were placed per Petri-dish containing one-quarter strength potato dextrose agar (PDA) for leaves infected with *Phomopsis leaf spot,* while those infected with frogeye leaf spot were placed on Petri-dish containing v-8 juice agar. After 7 days of incubation at room temperatures (27$\pm$ 2^{0} C), the organisms were transferred to new Petri-dishes containing PDA acidified with a drop of 25% lactic acid (Kmetz *et al*., 1975). Subsequent sub culturing was done until pure cultures of the organisms were obtained and maintained for use, by storing under cold temperature of 5^{o}C.

3.3 Pathogenicity test on seeds: Three organisms *Aspergillus niger, A.flavus* and *A. tamarii* were isolated from seeds and purified. Good quality soybean seeds without any damage were surface sterilized using 3% sodium hypochlorite solution for 2 minutes and rinsed in sterile distilled water and subsequently air dried. Each of the organisms isolated was used to artificially inoculate four hundred seeds of soybean cv. samsoy 2 (100 seeds/replicate) by dusting the seeds with spores of the organism. Each of the organisms used constituted a treatment and seeds which were not inoculated as control. Inoculated seeds were arranged in a completely randomised design and stored for 6 months under ambient temperatures and relative humidity of between 85 – 90%, after which they were assessed for percentage germination, physical damage on seeds and frequency of organism isolation after incubation. This was done by plating the seeds in moist blotters and incubating for 7 days after which the number of seeds that germinated were counted; the type of organisms isolated and the frequency of isolation was noted. Physical damage on seeds was assessed by examination of the seeds before incubating them and in another experiment; five organisms (*A. niger, A. flavus, Fusarium* sp., *Botryodiplodia theobromae* and

Phomopsis sojae) were used to inoculate soybean seeds (var samsoy 2) singly and in combinations (Table 4) and stored for ten months. There were a total of 17 treatments replicated 4 times. Four hundred soybean seeds for each treatment (100seeds/replicate) were sterilised with 3% sodium hypochlorite for 2 minutes and air dried before dusting with spores of the five organisms and arranged in completely randomised design. After 10 months of storage under ambient temperatures and relative humidity of 85-90%, the seeds were examined after 7 days of incubation for percentage germination and the frequency of organisms found growing on the seeds.

3.4 Pathogenicity test on seedlings: The inoculum for the pathogenicity tests were prepared by adding 10mls of sterile distilled water into pure cultures of *Aspergillus niger, A. flavus, Phomopsis sojae, Fusarium sp, Cercospora kikuchi*, and *Botryodiplodia theobromae* and scooping with sterile rubber teat to dislodge the conidia/spore. The conidia/spores suspension was filtered using sterile cheese cloth. One isolate was treated at a time to avoid contamination. The concentration of conidia/spore suspension for each isolate was determined using a haemocytometer and adjusted to 1 X 10^6 conidia or spore/ml for the experiment. A surfactant (Tween 20) was added to the conidia/spore suspensions to give a more uniform suspension of spores that will spread more evenly over the plant surface. Each of the isolates was used to artificially inoculate two week old seedlings and sterile water was used as control. The artificial inoculation of seedlings was done using spray guns to treat the leaves with the spore suspension until run-off. Sprayed plants were covered with transparent cellophane bags for 48 hours, so as to ensure 100% relative humidity. Subsequently, seedlings were sprayed with water daily to sustain the high humidity around them. There were five seedlings per pot and three pots per isolate or treatment which were replicated three times and repeated twice. Plants were arranged in a completely randomised design. Isolates were considered pathogenic if more than 60% of the inoculated seedlings were lodged, stunted, withered, dead or having any other symptoms after 14 days (Rizvi and Yong, 1996).

3.5 Pathogenicity test on leaves: Young and expanding, 4 weeks old healthy soybean leaves were used in the experiment. Five whole leaves from the soybean cv. samsoy 2 were used for each isolate and replicated four times and arranged in completely randomised design. They were sterilized using 3% hypochlorite solution and placed in Petri-dishes containing moist blotters. The experiment was repeated twice. Spray guns were used to spray the inoculum of the various isolates on the leaves in Petri-dishes. Inoculated leaves were incubated for seven days and isolates were considered pathogenic if more than 60% of the inoculated leaves had symptoms of disease (Rivzi and Yang, 1996).

3.6 Data analysis: Data were subjected to analysis of variance and the means separated using the Duncan's multiple range test at (P=0.05).

4 RESULTS

4.1 Isolated organisms: Seven organisms (*Aspergillus flavus, A. niger, A. tamarii, Phomopsis sojae, Cercospora kikuchii, Fusarium sp.* and *Botryodiplodia theobromae*), were isolated from the 12 varieties assessed (Table 1). The frogeye leaf spot disease caused by Cercospora sojina was observed in the field but the isolation of the organism in the laboratory using the blotter method failed. Analysis of variance showed significant differences in germination of seeds, and percentage infection (General infection) and frequencies of isolation of each organism. A higher percentage of infection (67.5%) was recorded on cv. TGX 1861-3F while cv. TGX 1844-4E had the lowest infection of 25%. TGX 1844-4E also had the highest germination percentage of 97.5% (Table 1). The results showed that there were varietal differences in the type and frequency of organisms attacking each of the varieties of soybean examined. *Aspergillus flavus* was the most frequently isolated organism from seeds in almost all the treatments except in TGX 1844-4E, followed by *A. niger*. Phomopsis sojae was the only organism that was successfully isolated from plants.

4.2 Pathogenicity test on seeds: Results of the pathogenicity test on seeds are presented in Tables 2 and 3. The organisms tested were all pathogenic on the seeds. The physical damage observed on the inoculated seeds, included brown stains, cracks on seeds and shrivelling of seeds. The cracking and shrivelling of seeds observed may be due to the physiological loss of water by the seeds during the period of storage and may not have been caused by the inoculated organisms. The control however had the greatest seed damage. When the seeds were examined after incubation as shown in table 3, there were significant differences in the frequency of isolation of the various organisms. The recovery rate of *A. niger* from inoculated seeds was 85% while the

recovery rate of *A. flavus* and *A. tamarii* were 75% and 52.5% respectively. There were significant differences in the germination of seeds inoculated with the three organisms. All inoculated seeds germinated more than the control by 35%, 22.5% and 17.5% for *A. flavus, A. tamarii* and *A. niger* respectively.

Table 4 shows the result of the percentage germination, percentage infection and frequency of occurrence of organisms isolated after 10 months of storage of soybean seeds artificially infected with one or two organisms. There were significant differences among the treatments in their percentage germination after 10 months of storage. Seeds treated with *A. flavus* germinated more than the control by about 17.5%. On the percentage infection of the seeds after 10 months of storage, there were significant differences recorded among the treatments. *A. flavus* was more frequently recovered from most of the treatments after 10 months of storage. The *Phomopsis sp.* isolated from plants and inoculated on seeds also caused the seeds to loose viability completely after 10 months of storage.

4.3. Pathogenicity test on seedlings and leaves. Results of the pathogenicity test on seedlings and leaves are presented in Tables 5 and 6. There were significant differences between the treatments in both years, on their disease incidence and severities. Among the organisms used, *Phomopsis sojae,* had the highest disease incidence in both years, both on seedlings and on leaves in Petri-dishes. All the organisms were pathogenic on the seedlings but *Phomopsis sojae* was more aggressive than others since it affected over 70% of the plants inoculated in both years. On leaves, *all the organisms* were also pathogenic. *Phomopsis sojae* infected about 83.3% of the seedlings and 100% of the leaves in Petri-dishes.

5 DISCUSSION

The organisms isolated from seeds of different varieties of soybean were similar to those reported previously (Sinclair and Backman 1989; Uwala and Ekpo 2000). Most of the organisms isolated in the experiment (e.g. *Phomopsis sojae*, *Fusarium sp.*, *Botryodiplodia theobromae* and *Cercospora kikuchi*) were pathogenic on the seeds and plants. Yang, (2000) stated that the many destructive pathogens of soybeans are seed borne and that the various organisms that infect soybean seeds can reduce seed viability and if such seeds are sown for production of subsequent crops reduced stands may result in lower yield or contribute to weed infestation due to lack of soybean competition. (Sinclair and backman 1989) stated that micro-organisms that invade and colonize seeds before harvest can reduce the yield and quality of the seed crop. In some cases mycotoxins produced by some organisms can cause problems for animals consuming soybean (Sinclair & Backman 1989, Okoro *et al.*, 2004). The most frequently isolated organisms were those belonging to the genus *Aspergillus*. *Aspergillus spp* have not been reported to cause any soybean disease in the field when infected seeds are planted. This was confirmed in this work, which clearly shows that *Aspergillus* spores artificially inoculated on the seedlings were not pathogenic. So *Aspergillus* species are not important as field disease problems. However the species was rather beneficial when seeds infected with it were stored. Tevet (1945) demonstrated that microrganisms, especially those belonging to the genus *Aspergillus* are important in the loss of viability of soybean in storage and probably are responsible for much of the heating of soybeans up to 45^{o}C. Sinclair and Backman (1989) also reported that *A. flavus* rapidly raises the temperature of stored soybean to 55^{0} C and brings about the deterioration of the seeds. The findings of this trial do not agree with that of Tevet (1945) and that of Sinclair and Backman (1989). Investigations have now shown that seeds artificially inoculated with *Aspergillus* spp germinated more than the control after 6 months of storage. Furthermore, *A. flavus* treated seeds were found to have germinated more than the control after 10 months of storage. This shows that when *A. flavus* was inoculated singly on the seeds it rather protected the seeds especially when the storage environment was conducive to avoid mouldiness and the soybean quantity stored was small (400 seeds each) and properly aerated to prevent over heating and also enhanced the germination. With this finding, it may become possible to use this organism as a biological control agent to control destructive soybean seed storage pathogens.

However, when the *Aspergillus* species were inoculated in combination with other organisms, the adverse effect on the seeds was devastating. All seeds inoculated with a combination of organisms lost their viability completely, six months into the storage period. This shows that the combination of organisms either had additive or synergistic effect on the seeds which caused more destruction on the seed viability. *Phomopsis sojae* reduced seed germination

more than the other organisms. This corroborates the findings of Kmetz *et al.,* (1974) who reported that *Phomopsis* spp may be the organisms most responsible for loss of viability of soybean seeds. *Phomopsis sojae* was also found to be pathogenic on the plants in the field. *P. sojae* infection on seed is important as it does not only affect seed quality, especially seed health and viability, but also has epidemiological implication of being carried to the field through seeds and where they become the source of primary inoculum. Yang (2000) stated that diseases caused by Phomopsis are seed borne. Infected seeds may be cracked and shrivelled and usually have a low germination rate and if these seeds are planted, it will result to low emergence or seedling blight. It is therefore important to protect soybean seeds from the devastating effect of *P sojae* by using seed dressing chemicals before planting. *Fusarium* spp and *Botryodiplodia theobromae* were not too virulent even though they affected the viability of seeds after 10 months of storage. However, since they recorded up to 50% incidence in the first year, when their pathogenicity were determined, they warrant close attention especially *Botryodiplodia* which caused total wilting of plants. *Cercospora kikuchii* was only important on the seeds where it caused purple seed stains.

The findings of this study have shown that some of the important diseases causing organisms of soybean in the Southern Guinea Savanna Zone of Nigeria include *Phomopsis sojae, Botryodiplodia theobromae, Fusarium* spp., *Cercospora kikuchii* and *Cercospora sojina* while *Aspergillus spp.* were found useful as biological control agents for soybean seed storage.

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TABLE 1: Fungal organisms isolated from seeds of 12 soybean varieties.

Varieties	Percentage Germination	Percentage Infection	**FREQUENCY OF ISOLATION (%)**						
			Aspergillus flavus	*Aspergillus niger*	*Phomopsis sojae*	*Cercospora kikuchii*	*Fusarium Sp.*	*Aspergillus tamarii*	*Botryodiplodia theobromae*
TGX 1852-3E	87.5c	57.5b	32.5ab	10.0b	7.5ab	2.5d	0.0c	0.0b	0.0b
TGX 1864-15F	95.0ab	35.0cde	22.5b	7.5bc	0.0c	2.5d	2.5bc	0.0b	0.0b
TGX 1861-3F	65.0de	67.5a	50.0a	17.5a	5.0bc	0.0d	2.5bc	0.0b	2.5a
TGX 1842-12E	52.5e	40.0c	35.0ab	7.5bc	5.0bc	0.0d	5.0ab	0.0b	0.0b
TGX 1856-1E	77.5bcd	32.5cde	15.0bc	7.5bc	2.5bc	5.0cd	2.5bc	0.0b	0.0b
TGX 1448-2E	75.0cd	27.5de	15.0bc	2.5cd	0.0c	7.5c	0.0c	2.5a	0.0b
TGX 1844-4E	97.5a	25.0e	2.5c	5.0cd	7.5ab	10.0bc	0.0c	0.0b	2.5a
TGX 1864-17E	72.5cd	37.5cd	20.0bc	10.0b	0.0c	7.5c	0.0c	0.0b	0.0b
M – 351	75.0cd	35.0cde	12.5bc	0.0b	5.0bc	12.5b	5.0ab	0.0b	0.0b
TGX 1835-5E	75.0cd	37.5cd	20.0bc	10.0b	2.5bc	5.0cd	0.0c	0.0b	0.0b
TGX 1869-13E	77.5bcd	32.5cde	22.5bc	10.0b	0.0c	0.0d	2.5bc	0.0b	0.0b
Samsoy 2	52.5e	55.0b	10.0bc	5.0bcd	12.5a	17.5a	7.5a	0.0b	0.0b

Means followed by the same letter within a column are not significantly different by the DNMRT at 5% level of probability.

TABLE 2: Mean frequencies of physical damage of soybean seeds inoculated with three organisms and stored for 6 months.

Treatments	MEAN FREQUENCIES OF DAMAGE SEEDS (%)				
	Brown stain	Purple stain	Cracked seeds	Green stain	Reduced/Shriveled seed
Aspergillus niger	0.00	0.00	5.00	0.00	0.00
Aspergillus flavus	2.50	0.00	27.50	0.00	12.5
Aspergillus tamarii	12.50	0.00	12.50	0.00	12.5
Control	41.78	8.19	11.14	7.75	12.95
LSD 0.05	8.32**	NS	NS	NS	2.20**

NS = Not Significant
** = Significant at both 5% &1% levels.

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TABLE 3: Frequency of isolation of organisms from seeds artificially infected with *a. niger, a. flavus, a. tamarii,* after 6 months of storage.

Treatments	Germination Percentage	FREQUENCIES OF ISOLATED ORGANISMS (%)				
		Aspergillus niger	*Aspergillus flavus*	*Aspergillus tamarii*	*Rhizopus sp.*	*Fusarium sp.*
Aspergillus niger	57.5	85.00	7.50	7.50	10.0	2.50
Aspergillus flavus	75.0	20.0	75.0	0.0	20.0	30.0
*Aspergillus tamar*ii	62.5	0.00	15.0	52.5	12.5	15.0
Control	40.0	32.5	85.0	7.50	15.0	7.5
LSD0.05	18.86**	4.41**	6.31**	2.21**	3.04**	4.82**

** = Significant at both 5% and 1% levels.

TABLE 4: Percentage germination, percentage infection and frequency of occurrence of organisms isolated from soybean seeds inoculated with some organisms singly and in combination after 10 months of storage.

Treatments	Germination Percentage	Percentage Infection	FREQUENCY OF ISOLATION (%)				
			Aspergillus flavus	*Fusarium Sp.*	*Aspergillus niger*	*Phomopsis sojae*	*Botryodiplodia theobromae*
A. Flavus + B. theobromae	0.0b	100.0a	100.0a	7.5bc	0.0c	0.0d	0.0c
A. Flavus + Fusarium sp.	0.0b	15.0e	0.0f	15.0ab	0.0c	0.0d	0.0c
A. Niger +Phomopsis sojae	0.0b	100.0a	40.0d	2.5bc	97.5a	15.0c	0.0c
A. Niger +Fusarium sp.	0.0b	57.5dc	0.0f	25.0a	0.0c	0.0d	0.0c
A. flavus + Phomopsis sojae.	0.0b	100.0a	72.5b	3.75bc	0.0c	0.0d	0.0c
A. Niger + A. flavus	0.0b	100.0a	75.0b	0.0c	23.33b	0.0d	25.0a
Fusarium sp + B. theobromae	0.0b	87.5ab	53.75c	16.25ab	0.0c	0.0d	27.5a
Fusarium sp + Phomopsis sojae	0.0b	98.75a	12.5e	26.25a	0.0c	75.0b	0.0c
A. niger + B. theobromae	0.0b	65.0c	22.5e	7.5bc	28.33b	0.0d	23.33a
Phomopsis sojae+ B. theobromae	0.0b	98.75a	22.5e	0.0c	20.0b	77.5b	6.25b
Phomopsis sojae	0.0b	16.25e	1.25f	0.0c	2.5c	11.25c	0.0c
Botryodiplodia theobromae	0.0b	27.5e	26.25e	6.25bc	28.75b	0.0d	0.0c
Aspergillus flavus	22.5a	80.0b	51.25c	0.0c	0.0c	0.0d	0.0c
Aspergillus _niger	0.0b	100.0a	57.5cb	0.0c	100.0a	0.0d	0.0c

Fusarium sp.	0.0b	5.0e	1.25f	2.5bc	0.0c	0.0d	0.0c
Control	5.0b	95.0a	85.0ab	12.5b	7.5cb	2.5d	0.0c
Phomopsis (Plant isolate)	0.0b	21.20c	5.75f	0.0c	1.5c	10.75c	0.0c

Means followed by the same letter within a column are not statistically different by the Duncan's New Multiple Range Test at 5% level.

TABLE 5: Disease incidence, severity and symptom expression on 2 weeks old seedlings inoculated with six isolates from infected seeds.

Treatments	Disease incidence %)		Disease Severity		Symptom expression
	Year 1	Year 2	Year 1	Year 2	
**Phomopsis sojae* at 6 WAP	83.32a	77.5a	0.67b	0.5a	Tiny leaf spots that grow and coalesce. Initially yellowish in colour and tan brown spots appearing later on top of the yellow ones
Aspergillus flavus	1.39d	1.33d	0.03c	0.01b	Organism seen growing on leaves without any symptom expression
Aspergillus niger	1.39d	1.67d	0.03c	0.05c	Organism seen growing on leaves without any symptom expression
Botryodiplodia theobromae	50.0b	5.0c	1.0a	0.1b	Wilting of the whole plant
Fusarium spp.	50.0b	20.0b	0.2c	0.1b	Drying of the leaves from the edges and curling.
Cercospora kikuchii	1.39d	1.67d	0.17c	0.1b	Leaf spots that are brownish.
Control	2.78c	1.33d	0.03c	0.01c	Symptoms of wilting and yellowing of leaves.

Means followed by the same letter within a column are not significant by Duncan's New Multiple Range Test at 5% level.
* Inoculated at 6WAP

TABLE 6: Disease incidence, severity and symptom expression on leaves in Petri-dishes inoculated with six isolates infected seeds.

Treatments	Disease incidence (%)		Disease Severity		Symptoms expression
	Year 1	Year 2	Year 1	Year 2	
Aspergillus flavus	21.41d	16.67d	0.5b	0.65b	Yellowing of leaves
Aspergillus niger	15.67e	16.67d	0.3c	0.20c	Yellowing of leaves
Botryodiplodia theobromae	83.81b	62.5bc	0.9ab	0.75a	Tiny greenish yellow spots on leaves.
Fusarium spp	60.0c	50.0c	0.5bc	0.5bc	Brownish spots with greenish yellow spots scattered, on leaves.
Phomopsis sojae	100.0a	100.0a	1.0a	1.0a	Leaf spots tiny and yellow in colour and brownish spots on top.
Cercospora kikuchii	98.33a	83.34a	1.25a	1.0a	Yellowing of leaves and leaf necrosis
Control	5.0f	12.08d	0.25c	0.3c	Yellowing of leaves

Means followed by the same letter within a column are not significant by Duncan's New Multiple Range Test at 5% level.