EFFECTS OF SINGLE AND COMBINED INFECTION OF *SOYBEAN MOSAIC VIRUS* AND *MELOIDOGYNE INCOGNITA* ON SOYBEAN AND REPLICATION AND PATHOGENICITY OF BOTH PATHOGENS

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Summary. Effects of single and combined infection of *Soybean mosaic virus* and *Meloidogyne incognita* in soybean cultivar TGm 80 and the multiplicity and infectivity of both pathogens in the crop were determined. A significant reduction in most growth and yield components of the soybean and of *Bradyrhizobium* root-nodules was observed. Combined infections significantly reduced the numbers of root galls, eggs and second-stage juveniles of the root-knot nematode compared to the numbers found with single infection with only the nematode. Combined infections in which virus application preceded that of the nematode caused the most significant inhibition of nematode growth and development.

Key words: *Glycine max*, Nigeria, pathogen interaction, root-knot nematode.

It has been demonstrated that certain plant-parasitic nematodes adversely affect their hosts. However, their major importance in plant disease development may be as host modifiers in combination with viruses and other pathogens (Bookbinder and Bloom, 1980; Gourd *et al.*, 1993; Iheukwumere *et al.*, 1996). In Nigeria, *Soybean mosaic virus* (SMV) and root-knot nematodes (*Meloidogyne* spp.) occur wherever soybean (*Glycine max* L.) is grown and constitute major constraints in the growth and production of the crop (Adesiyan *et al.*, 1990; Thottappilly, 1992; Atungwu and Afolami, 2001). Soybean has become increasingly important in Nigeria as a protein, oil and fibre source that is low in carbohydrate and nutrient dense. It is also considered a balanced diet for both humans and livestock (Anonymous, 1990; Atungwu and Afolami, 2001).

Although, the frequent occurrence of combined infection with *Soybean mosaic virus* and the root-knot nematodes in major soybean growing areas in Nigeria has elicited some attention (Iheukwumere *et al.*, 1996; Iheukwumere, 2006a,b), information on combined infections by both pathogens in the crop remains scanty. Lack of resistance in most cultivars continues to render them vulnerable to both SMV and the root-knot nematodes. Therefore, the present study aims at addressing the effects of single and combined infection of SMV and *Meloidogyne incognita* (Kofoid *et* White) Chitw. in simultaneous and successive infections of the soybean cultivar TGm 80, which is tolerant to SMV and susceptible to *M. incognita* race 2 (Iheukwumere *et al.,* 1995), a response that is different from that of other varieties of soybeans that were considered in the studies of Iheukwumere *et al.* (1996) and Iheukwumere (2006a,b). Although the cultivar TGm 80 was included in the study of Iheukwumere *et al.* (1996), their report investigated the effects of mixed infection only on the yield of the soybeans by using SMV and variable inocula levels of the nematode (10,000-20,000 eggs) applied simultaneously to the test plants. The study was to assess how each variety responded to such treatment, and there was no information on the replication and pathogenicity of the pathogens and the possible influence of one pathogen on the multiplication and development of the other (i.e. the virus or the nematode).

Iheukwumere (2006a,b) considered the varieties TGm 1784, resistant to both SMV (isolate SMV-10) and *M. incognita*, and Malayan, resistant to the nematode and highly susceptible to SMV-10 (Iheukwumere *et al.*, 1995), with the intention of evaluating their responses to infections in which both pathogens were inoculated onto each simultaneously and successively. A similar study (Iheukwumere *et al.*, 2007) was conducted on another variety of soybean, TGx 923-2E, that is susceptible to both the nematode and the virus. The present study addresses mono- and multi-pathogen effects of these two pathogens in simultaneous and successive infections of a soybean cultivar (TGm 80) that is tolerant to SMV and susceptible to the root-knot nematode (*M. incognita*) (Iheukwumere *et al.*, 1995). Also, the multiplication and pathogenicity of the virus and nematode and their interaction in the crop were investigated.

MATERIALS AND METHODS

Virus inoculum and inoculation. The virus isolate

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(SMV-10) used is a widely distributed strain of SMV in Nigeria and was made available by Dr. Thottappilly of IITA Ibadan. It was propagated and maintained under greenhouse conditions (29 ± 3 °C) at IITA by weekly transfer to susceptible seedlings of soybean cv. Malayan. Leaves of 7- to 10-day-old infected 'Malayan' seedlings were homogenized in 0.01 M pH 7.0 phosphate buffer in a ratio of 1:10 (W/V). The crude sap was manually inoculated on carborundum (600-mesh)-dusted leaves of test plants (TGm 80) in the greenhouse (29 ± 3 °C) according to standard methods (Walkey, 1991).

SMV-10 concentration in test plants. This was determined by a modification of the local lesion bioassay reported by Alam *et al.* (1990). Systemically infected trifoliate leaves of TGm 80 were homogenized in a sterile mortar and pestle in a ratio of 1 g tissue to 10 ml 0.01 M phosphate buffer (pH 7.0). The whole sap, further diluted to 1:20 in the same buffer, was used to elicit local lesions on the host *Chenopodium amaranticolor* Coste *et* Reyn preliminarily subjected to 12 hours of dark treatment before inoculation. The extracts were mixed with carborundum (600 mesh) and applied to the mature leaves of each six-week-old *C. amaranticolor* plant. The leaves were rinsed with distilled water immediately after inoculation. The plants were allowed to grow in 10-cm-diameter plastic pots in the greenhouse at 29 ± 3 °C. Plants whose leaves were abraded with buffer plus carborundum only were included as controls. Treatments were replicated five times in a completely randomized design and monitored for symptoms 7 days after inoculation. The numbers of lesions observed on the leaves of each plant were counted and recorded.

Nematode inoculum and inoculation. The species identity of the root-knot nematode, *M. incognita* race 2, obtained from roots of *Celosia argentea* L., was confirmed by perineal pattern morphology of the adult females according to Eisenback *et al.* (1981). The race was identified by the North Carolina differential host test (Hartman and Sasser, 1985). The nematode population was increased on *C. argentea* for eight weeks on benches in the greenhouse at 29 ± 3 °C. The eggs were extracted from the root system in 0.53% sodium hypochlorite (Hussey and Barker, 1973).

Plants were not watered the day prior to inoculation to avoid over-watering of the soil during inoculation, which might have caused loss of some eggs. Seedlings were inoculated with 10,000 eggs of the root-knot nematode using the trench method of Iheukwumere *et al.* (1995) and Iheukwumere (2006b). This consisted of making a shallow trench around the root rhizosphere of test plants and pouring the nematode suspension into it before closing the trench again.

Nematode root galling. This was evaluated by inspection of the washed root systems on a 0-5 scale (Taylor and Sasser, 1978) as follows: 0 = no galls; 1 = 1-2 galls; 2 = 3-10 galls; 3 = 11-30 galls; 4 = 31-100 galls; 5 = more than 100 galls.

Extraction of eggs and second stage juveniles (J2) of the nematode. Eggs were extracted from each root system using the sodium hypochlorite method of Hussey and Barker (1973). Juveniles were extracted from 200 cm^3 of soil per replicate using the pie-pan modification of Baermann's funnel method (Whitehead and Hemming, 1965; Iheukwumere, 2006b). Aliquots of the nematode water suspensions were placed in a counting dish and counted under a stereomicroscope at 40× magnification.

Soybean test plant. The soybean cv. TGm 80 is one of the improved cultivars of soybeans that have been released to Nigerian farmers after being tested for agronomic characters and yield in many ecological zones of

Table I. Effects of single and combined infections of *Soybean mosaic virus*, isolate SMV-10, and *Meloidogyne incognita* on shoot fresh and dry weights, root length and number of root nodules of soybean cv. TGm 80[1].

Treatment[2]	Shoot weight (g)		Root length (cm)	Number of nodules
	fresh matter	dry matter		
N	20.60a	7.68a	54.38a	34.90d
C	18.40b	6.94a	51.76a	59.70a
V	14.38c	5.47b	32.70b	41.50b
V + N	12.34d	2.53d	29.17c	37.70c
V + n	13.24cd	4.00c	25.62d	32.30e
N + v	10.50e	2.13d	24.80d	21.90f
S.D.	1.25	1.02	2.60	1.46

[1]Data are means of five replicates. Means followed by the same letter in each vertical column are not significantly different according to Duncan's multiple range test ($P = 0.05$).

[2]N = nematode; C = control, no virus or nematode inoculated; V = virus; V + N = virus and nematode inoculated simultaneously; V + n = virus inoculation followed by the nematode 7 days later; N + v = nematode inoculation followed by the virus 7 days later; S.D. = standard deviation; SMV-10 = *Soybean mosaic virus* isolate.

Nigeria by the IITA. It is high yielding and well adapted for growth in the country. Screening trials showed that it is tolerant to SMV-10 and susceptible to the root-knot nematode (Iheukwumere *et al.*, 1995), and this was the basis for its selection in this study.

Seed preparation and inoculation with Bradyrhizobium. Healthy seeds of TGm 80, obtained from IITA, were surface sterilized for 5 minutes in 1.05% sodium hypochlorite and rinsed for 5 minutes in distilled water (Koenning and McClure, 1981) prior to bacterization with *Bradyrhizobium* (Raut and Sethi, 1980). Bacterization was done by removing the nodules from healthy soybean roots, placing them on a 30-mesh screen and rinsing them with running tap water to wash off adhering soil particles. The nodules were removed from the screen and washed with a little soap in distilled water and then thoroughly rinsed. They were subsequently exposed to 95% ethanol for 10 seconds and afterwards immersed in 0.1% acidified mercury chloride ($HgCl_2$) for 5-10 minutes and washed in at least six changes of sterile distilled water (Tu *et al.*, 1970). Using a sterile glass rod, the nodules were crushed in a sterile Petri dish containing 10 ml of 5% sucrose solution, which served as sticker (Raut and Sethi, 1980). Inoculation of the seeds was by pipetting a 1 ml aliquot of the suspension onto each of the seeds placed in 9-cm-diameter Petri dish and gently shaken to evenly bacterize them with the suspension (Raut and Sethi, 1980; Ohki *et al.*, 1986; Iheukwumere, 2006a,b) prior to sowing.

Planting and treatment of test plants. Five seeds of the test plants were sown in 15-cm-diameter plastic pots with steam sterilized soil at five seeds per pot. Seedlings were later thinned to one seedling per pot at their VE stage (when the cotyledons were above the soil surface) (Fehr and Caviness, 1977). The treatments were: *i*) V = virus inoculated; *ii*) N = nematode inoculated; *iii*) V + N = virus and nematode inoculated simultaneously; *iv*) V + n = virus inoculated followed by that of nematode later; *v*) N + v = nematode inoculated followed by that of virus later; *vi*) C = control. The details of the treatments are as follows: V = plants were inoculated with crude sap of SMV-10 when unifoliate leaves had unrolled sufficiently so that the leaf edges were not touching (at VC stage) (Fehr and Caviness, 1977); N = plants were inoculated with 10,000 eggs of the nematode only at VC stage; V + N = plants were inoculated simultaneously with SMV-10 and 10,000 eggs of nematode at VC stage; V + n = plants were inoculated with SMV-10 at VC stage followed by inoculation with 10,000 eggs of nematode at fully developed leaves on unifoliate nodes (VI stage) 7 days later (VI = fully developed nodes) (Fehr and Caviness, 1977); N + v = plants were inoculated with 10,000 nematode eggs at VC stage followed by inoculation with SMV-10 at the (VI) stage 7 days later; C = control (no nematode nor virus were inoculated).

The experiment was arranged in a completely randomized design with five replicates per treatment on a greenhouse bench (29 ± 3 °C) at IITA. Plants were watered as necessary and inspected weekly for expression of symptoms. The experiment was terminated 97 days after planting when 95% of the pods had reached the mature stage (Fehr and Caviness, 1977) and the following variables were evaluated: shoot fresh and dry weights, root length, number of nodules, root fresh and dry weights, number of pods, pod fresh and dry weights, root galls, number of eggs and juveniles and SMV-10 local lesion counts.

Statistical analysis. Data obtained were subjected to analysis of variance and means separated according to Duncan's Multiple Range Test at $P = 0.05$. Data from gall rating, numbers of eggs and juveniles, and local lesion host counts were square root transformed before analysis of variance.

RESULTS

Plant growth and yield. The plant growth and yield responses to single and mixed infections of SMV and root-knot nematode are represented by the fresh and dry weights of the shoot and root including the seed yield of the plant. There were significant differences among the shoot fresh weights of N, C, V, V + N and N + v treatments. No significant difference in shoot fresh weight was detected between V + N and V + n treatments (Table I). Data on mean shoot dry weight showed no significant difference between N and C and these treatments had significantly higher dry weights than all the other treatments. The mean shoot dry weight followed this order of decreasing magnitude: N or C, V, V + n, V + N and N + v, with significant differences at $P = 0.05$ (Table I). For mean root length, no significant differences were found between N and C or between V + n and N + v. Root lengths in mixed infections were significantly less than in C, N and V (Table I). There were significant differences in the mean number of nodules among all the treatments: the control plants had the highest number of nodules, followed by V, V + N, N, V + n and N + v (Table I).

Mean root fresh weights showed no significant difference between N and C or among V, V + N, V + n and N + v, but V, V + N, V + n and N + v had significantly lower root weights than N or C (Table II). The root dry weights for N and C also were not significantly different but there were significant differences among V, V + N, V + n and N + v (Table II). The mean number of pods was not significantly different between N and C, or V and V + N, or V + n and N + v but the number of pods in N and C was significantly greater than in the other treatments (Table II). The N and C treatments had significantly higher pod fresh and dry weights than V, V + N, V + n and N + v.

Multiplication and pathogenicity of the root-knot nematode and the virus. The mean numbers of root galls

Table II. Effects of single and combined infections of SMV-10 and *M. incognita* on root fresh and dry weights, number of pods, and pod fresh and dry weights of soybean cv. TGm 80[1].

Treatment[2]	Root weight (g)		Number of pods	Pod weight (g)	
	fresh matter	dry matter		fresh matter	dry matter
N	13.08a	5.16a	28.50a	41.90a	22.30a
C	12.30a	4.90a	27.90a	40.00a	21.36a
V	8.98b	3.52b	20.10b	28.76b	15.74b
V + N	8.48b	2.74c	18.90b	25.88c	14.17c
V + n	7.56b	1.96d	15.50c	19.62d	12.60d
N + v	7.82b	1.18e	14.30c	15.10e	11.03e
S.D.	1.25	0.56	2.55	1.80	1.11

[1]Data are means of five replicates. Means followed by the same letter in each vertical column are not significantly different according to Duncan's multiple range test ($P = 0.05$).
[2]N = nematode; C = control, no virus or nematode inoculated; V = virus; V + N = virus and nematode inoculated simultaneously; V + n = virus inoculation followed by the nematode 7 days later; N + v = nematode inoculation followed by the virus 7 days later; S.D. = standard deviation; SMV-10 = *Soybean mosaic virus* isolate.

on plants with mixed infections, regardless of whether the plants had been inoculated simultaneously or successively, were significantly lower than the number on plants infected with only the nematode (Table III). The same trends were also observed for the numbers of eggs and the numbers of juveniles. Nematode reproduction and pathogenicity, measured by the numbers of eggs and the degree of galling, were lower when plants were also infected with the virus. There were no significant differences in the concentration of the virus in plants with single and multi-infections (Table III).

DISCUSSION

Infection with both pathogens, irrespective of whether it was simultaneous or successive, generally resulted in greater reductions in growth and yield components of the soybean than infection with just one of the pathogens. The parasitic activities of the virus and the nematode could have distorted and possibly disrupted normal physiological and metabolic processes in the soybean, thereby reducing growth and development. Other possible reasons for this reduction are that single infection by either SMV or the nematode can cause decreased nitrogen fixation in the plant, photosynthesis may be decreased, and there may be increased energy consumption through increased respiration and an imbalance in auxin levels in the plant (Husain *et al.*, 1985; Hussey, 1985; Ohki *et al.*, 1986).

In general, no significant differences in the various growth and yield parameters were detected between the

Table III. Effects of single and combined infections of SMV-10 and *M. incognita* on growth and pathogenicity of the *Soybean mosaic virus* and the root-knot nematode in soybean cv. TGm 80[1].

Treatment[2]	Gall index[3]	Number of eggs	Number of J2[4]	Number of SMV lesions
N	3.50a	5523.80a	83.00a	0.00b
C	0.00d	0.00c	0.00e	0.00b
V	0.00d	0.00c	0.00e	12.00a
V + N	2.30b	1423.00b	57.00b	12.00a
V + n	1.60c	884.00c	22.20d	12.00a
N + v	2.50b	1485.00b	45.60c	12.00a
S.D.	0.49	435.49	2.88	1.14

[1]Data are means of five replicates. Means followed by the same letter in each vertical column are not significantly different according to Duncan's multiple range test ($P = 0.05$).
[2]N = nematode; C = control, no virus or nematode inoculated; V = virus; V + N = virus and nematode inoculated concomitantly; V + n = virus inoculation followed by the nematode 7 days later; N + v = nematode inoculation followed by the virus 7 days later; S.D. = standard deviation; SMV-10 = *Soybean mosaic virus* isolate.
[3] Gall Index: 0 = no galls; 1 = 1-2 galls; 2 = 3-10 galls; 3 = 11-30 galls; 4 = 31-100 galls; 5 = more than 100 galls (Taylor and Sasser, 1978).
[4]J2 = second-stage juveniles.

control and single infection with the nematode, even though previous rating had indicated that this variety was moderately susceptible to the nematode (Iheukwumere *et al*., 1995). In fact, in some cases, the growth and yield values of the nematode-infected plants were slightly higher than those of the controls. A possible reason for this observation may be a low infection rate, which frequently stimulates growth in nematode infected plants (Hussey, 1985; Okorocha and Ezeigbo, 1992). The significant reductions observed in single infection with the virus occurred even though the soybean TGm 80 had been rated tolerant to single infection by the virus (Iheukwumere *et al*., 1995). The implication of these findings with single infections with either pathogen in this study and that of Iheukwumere *et al*. (1995) is that tests that measure resistance by relying on visual scores or gall counts may give incomplete information and may, in fact, fail to detect certain resistance or tolerance phenomena in "susceptible" lines (Bookbinder and Bloom, 1980) and vice-versa.

Combined infections significantly reduced nematode multiplication and infectivity, which indicates that the virus probably induced changes in the plant that hindered growth and development of the nematode adversely. Such antagonism would account for the suppression of egg production by the nematode, the reduction of the soil nematode population, and the severity of the galling produced (Goswami and Chenulu, 1974). Since any change caused by an organism (or infective agent) in one part of a plant can affect the physiology of other parts of the plant, and thus may act at some distance from the source (Norton and Niblack, 1991), the virus is a possible cause of physiological changes that inhibited nematode development in the soybean plants. The inhibitory effect of SMV-10 on the nematode in this soybean has been similarly demonstrated in other crops by other studies (Fritzische, 1970; Naqvi *et al*., 1977; Alam *et al*., 1990).

Although there were no significant differences in the mean degree of root galling and number of nematode eggs between V + N and N + v, the treatment in which virus infection preceded that of the nematode (V + n) caused the greatest reductions in these development indices of the nematode. The absence of significant differences in nematode development when pathogen introduction was simultaneous (V + N) or where nematode infection preceded that of virus (N + v) suggests that there was insufficient time for the virus to alter host physiology sufficiently to cause significant differences between the two treatments.

The antagonistic influence of the virus on the nematode could have prevented it from exerting any noticeable effect on the multiplication or infectivity of the virus, and hence there were no significant differences in the mean number of local lesions formed in single and mixed infections with both pathogens. Results similar to this finding have been reported in other host plants (Goswami *et al*., 1974) and other varieties of soybeans (Iheukwumere, 2006a,b).

It is clear from this study that combined infection of SMV-10 and the root-knot nematode caused a significant decrease in the growth and yield of this soybean, with the virus having an inhibitory effect on nematode growth and development. This phenomenon was also noted in another soybean cultivar (TGx 923-2E) rated susceptible to this nematode and SMV-10 (Iheukwumere *et al*., 2007). However, to contend with these growth and yield reductions, breeding for resistance to both pathogens in the crop is suggested.

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