GROWTH AND PHYSIOLOGICAL RESPONSES OF OKRA (*ABELMOSCHUS ESCULENTUS* (L.) MOENCH) TO SIMULATED ACID RAIN AND ROOT-KNOT NEMATODE (*MELOIDOGYNE INCOGNITA*)

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Summary. This investigation was undertaken to ascertain the single and combined effects of simulated acid rain (SAR) and the root knot nematode (RKN), *Meloidogyne incognita*, on okra. Root knot nematode inoculated and uninoculated plants were intermittently exposed to SAR (pH 4.0). In combined treatment, plants were exposed to SAR either concomitantly with or a week after inoculation. SAR exposure either alone or in combination with nematodes caused white-to-tan spots on the abaxial and adaxial surfaces of leaves. Single and combined treatments with *M. incognita* and SAR significantly suppressed pigment synthesis, shoot and root dry weights and yield of okra. Reducing and non-reducing sugars were depleted to varying degrees in all treatments. Non-reducing sugars were reduced to a greater extent than were the reducing sugars. This effect was more pronounced when the plants were treated with SAR and infected with root-knot nematode. SAR exposure, either alone or in combination with *M. incognita*, resulted in accumulation of soluble phenols, possibly as an induced defence mechanism against abiotic and biotic stresses. The fecundity of *M. incognita* was markedly suppressed in plants exposed to SAR as compared with those infected with *M. incognita* alone. The results are discussed in the light of pathological and physiological responses of plants to pathogens. Apparently, SAR and the nematode interacted additively and synergistically (reduction of chlorophyll and increase of soluble phenol contents) as their combination caused greater damage to okra than single treatments, especially when there was a post-inoculation exposure to SAR.

Keywords: Foliar injury, leaf chlorophyll content, nematode suppression, phenol concentration, sugar concentration.

Acid rain is harmful to terrestrial and aquatic ecosystems (Wellburn, 1989; Heij and Erisman, 1997). Investigations of the influence of acid rain on plant pathogenic nematodes have yielded variable results (Khan and Khan, 1994a). With regards to the effect on plants, it is known that acid rain alters leaf physiology (Evans, 1982; Haines *et al.,* 1985) which in turn might influence the response of plants to pathogens such as nematodes (Bolla and Fitzsimmons, 1988; Asai and Futai, 2001). Heagle *et al.* (1983) investigated the effect of SAR in the field on growth and yield of soybeans, soil chemistry and nematode populations. Although SAR at pH 2.8 caused slight foliar injury, plant growth, pod yield, seed protein content and populations of parasitic nematodes remained unaffected. Demowska (1993) demonstrated marked changes in the composition and diversity of nematode populations in response to long-term artificial acid rain. Application of simulated acid rain (SAR) in combination with root-knot nematode, *Meloidogyne incognita* (Kofoid *et* White) Chitw., substantially retarded growth, yield and pigment synthesis of tomato plants and also suppressed galling and fecundity of the nematode (Khan and Khan, 1994a).

Plants have evolved various mechanisms to defend themselves from pathogen attacks. Among several defence mechanism one is the accumulation of free phenols in the roots (Nicholson and Hammerschmidt, 1992; Ellard-Ivery and Douglas, 1996). Sitaramaiah and Singh (1978) demonstrated increased concentrations of free phenols in response to infection by root-knot nematode *M. javanica* (Treub) Chitw. Free phenol content also is known to increase in response to abiotic stresses (Dixon and Paiva, 1995; Abreu and Mazzafera, 2005; Olenchenko and Zagoskina, 2005).

This investigation attempts *a*) to examine the effect of simulated acid rain (SAR) either alone or in conjunction with the inoculation of okra with the root-knot nematode, *M. incognita*, on growth, yield, pigment synthesis, levels of reducing and non-reducing sugars and soluble phenol accumulation, and *b*) to assess the effect of SAR on nematode soil population, root-knot development and reproductive potential of *M. incognita.* The goal was to assess to what extent abiotic stress, such as SAR, can alter the normal physiology of okra and whether SAR-exposed plants undergoing physiological changes can alter the pathogenic response to *M. incognita*.

MATERIALS AND METHODS

Culture and treatments. Two-week-old okra (*Abelmoschus esculentus* (L.) Moench.) cv. Mirpurkhas-1 seedlings raised in steam-sterilized soil were planted individually in 18 cm diameter plastic pots (2000 cm^3) containing 1.75 kg sterilized sandy loam soil (72% sand, 18.5% silt and 9.5% clay; pH 7.8; organic matter 0.3%). Okra seedlings were allowed to establish in pots for three weeks before being treated. To the soil in each inoculated pot were added 2,000 freshly (within 48 h) hatched second stage juveniles (J_2) of *M. incognita* from

aubergine roots on which they were cultured from a single egg mass. Four small holes were made around the plant and nematodes suspended in water (10 ml) were added to the holes (2.5 ml suspension/hole), which were subsequently re-closed with soil.

SAR solution was prepared in accordance with Capron and Hutchinson (1986). The SAR solution contained per litre: 20 µmol KOH, 27 µmol $CaSO_4$, 10 µmol NaOH, 27 µmol $FeCl_3$, 0.1 µmol $PbCl_2$, 1.5 µmol $ZnCl_2$, 0.18 µmol $MnCl_2$ and 0.15 µmol $CuCl_2$. SAR solution was adjusted to pH 4.0 using a mixture of 50 µmol/l H_2SO_4 and HNO_3, while controls received deionized distilled water. The exposure to SAR was in the form of a twice per week shower of 150 ml SAR solution per plant. The entire plant was exposed to a light spray using a small hand-held sprinkler (1.5 l capacity). The soil moisture content was determined after the first SAR-treatment and found to be 26.9% of dried weight, and the amount of soil exposed to the treatment was roughly 500 cm^3 (top layer).

Simulated acid rain (SAR) treatment was either applied at the same time as (concomitant treatment) or subsequent to nematode inoculation (post-inoculation treatment). In case of post-inoculation treatment, seedlings were exposed to SAR on the 6th day after nematode inoculation; in the concomitant treatment, SAR exposure and root-knot inoculation were done simultaneously. Treatments and controls were replicated five times and completely randomized on a greenhouse bench. Day/night temperature regime in the plastic-house was 32/25 °C with a 14 h photoperiod. Natural light was supplemented by light from Philips 200 watt light bulbs (2400-2800 lumen each light bulb). The light in the plastic-house was comparable to natural light. Relative humidity during the experiment varied between 50-60%. Plants were irrigated daily with 225 ml of tap water. Each pot was provided with 100 mg urea as the soil was low in nitrogen. The plants were exposed to the designated SAR concentrations twice a week for a total of 15 and 16 exposures for post-inoculation and concomitant exposures respectively, as already explained. The soil pH after SAR application was 7.6.

Assessment of plant growth and nematode reproduction. Plants were harvested at 56 days after inoculation and growth components, including root and shoot dry weights (70 °C for 24 h) and okra yield (fresh weight of fruits) were recorded. The numbers of galls on the roots were recorded and egg masses produced on the entire root system were counted using a hand lens. Fecundity (number of eggs per egg mass) was determined at the time of harvest by excising 10 egg masses from each okra root. Root-knot nematode J_2s were extracted from a 250 cm^3 soil sub-sample per pot using a modified Baerman funnel technique and counted (Rodriguez-Kabana and Pope, 1981).

Estimation of chlorophyll and visual estimation of the symptoms. Chlorophyll a and b contents of leaves were estimated by extraction from 1 g of fresh leaves from each plant separately in 100 ml of 80% acetone at 20 and 40 days after treatment. The extract was filtered and optical densities were recorded at 663 and 645 nm for estimating chlorophyll a and b, respectively (Arnon, 1949).

Estimation of sugar content. Sugars were estimated at 10, 20, 30, 40 and 50 days following treatments. Total soluble sugars other than starch were extracted from fresh leaf material according to the procedure of Cerning and Guilhot (1973). Soluble sugars were determined spectrophotometrically using anthrone reagent following the method of Yemm and Willis (1954). Reducing sugars were determined using copper reagents in accordance with Nelson-Somogyi's modified method (Somogyi, 1945; Marais *et al*., 1966). The amount of non-reducing sugars was calculated as the difference between total soluble sugars and reducing sugars.

Determination of soluble phenols. Soluble phenol contents were also ascertained at 10, 20, 30, 40 and 50 days after treatments. Levels of soluble phenols in roots were determined as described by Dihazi *et al*. (2003). Root tissues (400 mg) were taken from each plant and homogenized in an ice bath with 2 ml 80% methanol v/v. The homogenate was centrifuged three times at 7,000 g for 3 min. One hundred µl of the supernatant was added to Folin-Ciocalteau reagent (0.5 ml) and saturated sodium carbonate. The mixture was incubated at 40 °C for 30 min. and the absorbance of the developed blue colour was read at 750 nm. Catechol was used as standard. The relative concentration of soluble phenols was expressed as µg/mg fresh weight.

Statistcal analysis. Data were subjected to statistical analysis following Zar (1999). One-way analysis of variance (ANOVA) was performed for shoot and root dry weights, fruit weight, RKN population in soil, galls per root system, egg masses per root system and fecundity. Factorial analysis of variance (FANOVA) was carried out for all the biochemical variables. Least significant difference (LSD) at P = 0.05 was employed as post-hoc test.

RESULTS

Foliar injury and leaf chlorophyll content. SAR-treatments and combined treatments of SAR and root-knot nematode inoculation caused white-to-tan irregular lesions on both the abaxial and adaxial surface of okra leaves. The foliar symptoms were more pronounced in joint treatment (plants treated both with SAR and nematode inoculation, concomitantly or post-inoculation), particularly in post-inoculation exposure to SAR. In plants inoculated with root-knot nematode alone, such

symptoms were not visible; however, slight chlorosis on some leaves and stunting of plants could be observed at about 17-20 days after inoculation. Chlorophyll a and b were both significantly decreased by SAR-treatment alone ($P<0.01$) relative to uninoculated controls (Table I). SAR, whether applied in conjunction with RKN as a post-inoculation or a concomitant treatment, dramatically ($P<0.001$) reduced chlorophyll a and b contents compared to controls. Chlorophyll a and b contents were also found significantly ($P<0.05$) lowered in the leaves of plants inoculated with root-knot nematode only as compared with uninoculated plants. Chlorophyll a was generally more and synergistically affected by the treatments than chlorophyll b. Furthermore, the effect of SAR alone on chlorophyll content was comparatively greater than that due to root-knot inoculation alone.

Sugar concentration. Reducing sugars declined significantly from 20 days onwards following treatment by SAR alone ($P<0.05$) relative to controls (Table II). Com-

Table I. Effect of simulated acid rain (SAR) exposure, SAR and *Meloidogyne incognita* inoculation (concomitant or post-treatment) or *M. incognita* inoculation alone on chlorophyll a and b content (mg/g FW) of okra leaves at 20 and 40 days after treatment (mean ± standard deviation).

Treatment	Day	Chlorophyll a	Chlorophyll b
Uninoculated (Control)	20	0.651 ± 0.12	0.517 ± 0.10
	40	0.633 ± 0.07	0.506 ± 0.05
SAR	20	0.580 ± 0.09	0.453 ± 0.09
	40	0.535 ± 0.14	0.435 ± 0.12
Post-inoculation exposure to SAR	20	0.460± 0.06	0.442 ± 0.08
	40	0.464 ± 0.11	0.438 ± 0.05
Concomitant exposure to SAR	20	0.469 ± 0.06	0.454 ± 0.09
	40	0.472 ± 0.09	0.446 ± 0.13
Root-knot inoculation alone	20	0.612 ± 0.07	0.464 ± 0.07
	40	0.596 ± 0.04	0.482 ± 0.08

$LSD_{0.05}$ (treatments) = 0.016; $LSD_{0.05}$ (time) = 0.011.

Table II. Effect of simulated acid rain (SAR), SAR and *M. incognita* inoculation (concomitant or post-treatment) or *M. incognita* inoculation alone on reducing sugar content of okra leaves (mg/g fresh weight) at various time periods after treatment (mean ± standard deviation).

Treatment	Days				
	10	20	30	40	50
Uninoculated (control)	21.5 ± 2.2	20.8±1.9	19.0 ± 2.4.	21.6 ± 2.6	19.2 ± 1.8
SAR	19.8 ± 1.5	18.0 ± 2.4	16.8 ± 0.9	17.6 ± 0.7	16.4 ± 1.8
Post-inoculation exposure to SAR	19.6 ± 1.8	16.2 ± 1.9	15.7 ± 1.6	16.5 ± 0.9	15.2 ± 1.5
Concomitant exposure to SAR	19.2 ± 1.7	17.4 ± 1.5	16.6 ± 0.8	17.3 ± 0.9	15.5 ± 1.0
Root-knot inoculation alone	20.9 ± 2.6	17.8 ± 1.0	17.5 ± 1.6	15.8 ± 1.7	16.2 ± 1.4

$LSD_{0.05}$ (treatments) = 0.648.

Table III. Effect of simulated acid rain (SAR) exposure, SAR and *M. incognita* inoculation (concomitant or post-treatment) or *M. incognita* inoculation alone on non-reducing sugar content of okra leaves (mg/g fresh weight) at various time periods after treatments (mean ± standard deviation).

Treatment	Days				
	10	20	30	40	50
Uninoculated (control)	12.4 ± 1.2	12.6 ± 0.9	11.5 ± 0.8	10.4 ± 1.0	10.8 ± 0.9
SAR	9.4 ± 0.7	8.8 ± 0.8	6.4 ± 0.6	6.9 ± 0.8	7.2 ± 0.6
Post-inoculation exposure to SAR	8.7 ± 0.5	6.0 ± 0.7	4.1 ± 0.4	3.8 ± 0.5	4.2 ± 0.3
Concomitant	9.2 ± 0.6	7.4 ± 0.9	5.2 ± 0.7	4.4 ± 0.4	6.0 ± 0.8
Root-knot inoculation alone	9.6 ± 0.9	9.3 ± 1.2	7.2 ± 0.5	7.8 ± 0.8	7.5 ± 0.9

$LSD_{0.05}$ (treatments) = 0.339.

bined treatment with SAR and RKN, both concomitant and post-inoculation, caused significant ($P<0.01$) reduction in the level of reducing sugars compared to uninoculated controls at all the sampling periods. RKN inoculation of okra also resulted in a significant ($P<0.05$) decrease from 20 days post-inoculation onwards. Among all treatments, the greatest reduction in reducing sugars occurred when the plants were exposed post-inoculation to SAR. This was followed closely by plants exposed concomitantly with SAR and nematode inoculation. Non-reducing sugars also were significantly ($P<0.05$) decreased in plants exposed to SAR as compared with controls at all sampling periods (Table III). Combined treatment showed a more dramatic reduction in the level of non-reducing sugars over the controls than did SAR exposure alone (P at the most 0.01). Post-inoculation exposure to SAR had a more drastic effect in this respect than the concomitant exposure. Although RKN infection alone also depleted non-reducing sugars relative to controls, the reduction was not as great as that caused either by SAR treatment alone or by the combined treatments.

Soluble phenol concentration. Treatment of okra plants with SAR, RKN or a combination of SAR and nematode inoculation resulted in high phenol accumulation in okra roots compared with the controls (P at the most 0.05) across all sampling periods (Table IV). Phenol accumulation reached maximum levels in post-inoculation exposure to SAR; on the 30th and 40th day following inoculation the level of soluble phenols was nearly double that of uninoculated controls. Phenol accumulation was also greatly and synergistically enhanced in concomitant exposure but the concentration was slightly lower than with post-inoculation exposure to SAR. Root-knot nematode inoculation or SAR-exposure alone also caused marked

Table IV. Effect of simulated acid rain (SAR), SAR and *M. incognita* inoculation (concomitant or post-treatment) and *M. incognita* inoculation alone on soluble phenol content (µg/g fresh weight) of okra roots at various time periods after treatment (mean ± standard deviation).

Treatment	Days				
	10	20	30	40	50
Uninoculated (control)	354 ± 12	347 ± 14	351 ± 19	345 ± 16	353 ± 17
SAR	352 ± 10	396 ± 13	415 ± 16	411 ± 15	422 ± 19
Post-inoculation exposure to SAR	389 ± 13	545 ± 17	697 ± 21	681 ± 19	598 ± 22
Concomitant exposure to SAR	380 ± 15	532 ± 13	646 ± 21	585 ± 16	626 ± 14
Root-knot inoculation alone	339 ± 13	396 ± 16	413 ± 19	486 ± 16	462 ± 17

$LSD_{0.05}$ (treatments) = 24.72.

Table V. Effects of simulated acid rain (SAR), SAR and *M. incognita* inoculation (concomitant and post-treatment) and *M. incognita* inoculation alone on dry matter production and yield of okra at 56 days after treatment (mean ± standard deviation).

Treatment	Dry weight of shoot (g)	Dry weight of root (g)	Fruit fresh weight per plant (g)
Uninoculated control	18.86 ± 1.18	2.66 ± 0.23	136.52 ±5.28
SAR	16.53 ± 1.34	2.35 ± 0.15	115.04 ± 5.96
Post inoculation exposure to SAR	14.12 ± 1.53	2.13 ± 0.18	85.57 ± 6.21
Concomitant exposure to SAR	14.55 ± 1.65	2.22 ± 0.17	97.42 ± 7.45
Root-knot inoculation only	16.60 ± 1.68	2.47 ± 0.19	116.71 ± 7.86
$LSD_{0.05}$	2.22	0.26	19.42

increase in soluble phenol content over the controls throughout the observation period, although such levels were less than in the joint treatment.

Dry matter production and yield. Simulated acid rain (SAR) alone significantly ($P<0.05$) suppressed shoot and root growth (dry weight) of okra plants compared with controls. Greater reductions in shoot and root dry weights ($P<0.01$) were caused in joint treatment of SAR and root-knot inoculation (Table V). Shoot growth was reduced by 20.1 and 18% and root growth by 20.1 and 18% by post-inoculation and concomitant exposure to SAR respectively. Root-knot nematode inoculation alone significantly ($P<0.05$) decreased shoot dry weight by 7.7% compared to controls but root dry weight did not differ significantly from the controls. Fruit weight (yield) was significantly reduced (P at the most 0.05) by all treatments. SAR exposure and RKN alone reduced the yield by 15.7 and 14.5%, respectively. The combined treatment of SAR and root-knot nematode inoculation resulted in drastic yield reductions of 37.2 and 28.6% with post-inoculation and concomitant exposures, respectively.

RKN population. The soil nematode population did not change significantly in the combined treatment with SAR, either post-inoculated or concomitant exposure, compared to root-knot nematode inoculation alone, although gall formation by *M. incognita* on okra roots was significantly ($P<0.05$) reduced with post-inoculation exposure to SAR compared to plants inoculated with RKN alone (Table VI). Concomitant or post-inoculation SAR treatment significantly suppressed the number of egg masses/root system as well as nematode fecundity ($P<0.05$) compared to plants that received RKN inoculation treatment alone.

DISCUSSION

Simulated acid rain at pH 4.0 caused characteristic white-to-tan irregular lesions on both of the surfaces of okra leaves and this was associated with decreased chlorophyll content. This is in accordance with earlier results of Evans (1982), Percy (1986) and Khan and Khan (1994a). In both of the joint treatments of *M. incognita* and SAR (concomitant and post-inoculation),

Table VI. Influence of combined treatment of simulated acid rain (SAR) and *M. incognita* inoculation (post-treatment and concomitant) or *M. incognita* inoculation alone on soil nematode population densities, galling intensity, egg masses/root system and fecundity (mean ± standard deviation).

Treatment	Nematode population in 250 cm^3 soil	Gall per root system	Egg masses per root system	Fecundity
Post-inoculated exposure to SAR	1856 ± 164	75 ± 12	41 ± 6	159 ± 16
Concomitant exposure to SAR	1832 ± 178	87 ± 14	47 ± 9	185 ± 18
Root-knot inoculation only	2257 ± 175	95 ± 17	68 ± 10	248 ± 25
$LSD_{0.05}$	389	18	14	28

the response of okra to the combination, as seen by more pronounced leaf symptoms and greater reduction in leaf pigments, was greater than in plants receiving *M. incognita* inoculation only. This response was more pronounced with post-inoculation exposure to SAR than with concomitant exposure. Khan and Khan (1994a) demonstrated greater injury and inhibition of pigment synthesis in post-inoculation treatment relative to SAR treatment alone at pH 6.8. The uptake of SAR may have been enhanced in the nematode-infected plants because of the wider stomatal pores that result in some plants following infection with RKNs (Odihrin, 1971; Khan and Khan, 1994b). Leaf chlorosis and lesions would compromise the photosynthetic capability of plants, which would consequently result in growth reduction.

Both reducing and non-reducing sugars declined substantially in SAR exposure alone, which agrees with the findings of Ferenbaugh (1976) who found reduced carbohydrate production in *Phaseolus vulgaris* L. following SAR treatment. Both reducing and non-reducing sugars declined markedly when SAR exposure was given in combination with root-knot inoculation but non-reducing sugars were the more drastically depleted. This corroborates the earlier results of Bolla and Fitzsimons (1988), who recorded a remarkable decrease in non-reducing sugars in SAR-treated pine seedlings inoculated with pine-wilt nematode [*Bursaphelenchus xylophilus* (Steiner *et* Buhrer) Nickle].

Simulated acid rain and root-knot nematode inoculation alone or in combination with SAR resulted in the accumulation of soluble phenols in okra roots. It has been established that phenol metabolism is activated in plants as a reaction to pathogens (Nicholson and Hammerschmidt, 1992; Metraux and Raskin, 1993; Ellard-Ivery and Douglas, 1996; Dihazi *et al.,* 2003) and to various types of abiotic stresses, including acid rain (Dixon and Paiva, 1995; Abreu and Mazzafera, 2005; Olenchenko and Zagoskina, 2005; Ganeva and Zozikova, 2007). Meyers (1988) demonstrated that pines respond to inoculation with the nematode *B. xylophilus* by initiating a hypersensitive response that includes synthesis of phenolic compounds and terpenes. Sitaramiah and Singh (1978) reported enhanced levels of phenolics in plants in response to *M. javanica* infection. Similarly, Badra and Elgindi (1979) reported accumulation of phenols in citrus plants infected with citrus nematode *Tylenchulus semipenetrans* Cobb. Our results, showing increased production of free phenols in response to SAR exposure or *M. incognita* inoculation alone or in joint treatments of SAR and *M. incognita*, confirm the role of these compounds in activating plant defence system under varied biotic and abiotic stresses. The production of free phenols was much accentuated in joint treatments, particularly with post-inoculation exposure to SAR. Increased synthesis of phenols would require precursors of simple carbohydrates derived from glycolysis and pentose phosphate shunt for the synthesis of various phenolic acids via the shikimic acid pathway (Vermerris and Nicholson, 2006). This need for precursors for the biosynthesis of phenolics suggests diversion of *de novo* synthesized and stored carbohydrates (non-reducing sugars) away from pathway of energy production to pathways for synthesis of a chemical response.

Acid rain is known to alter leaf physiology, reduces the ability of plants to resist pathogens (Haines *et al.*, 1985; Bolla and Fitzsimons, 1988) and might, therefore, influence the response of okra to RKN. SAR treatment alone resulted in depletion of sugars, particularly non-reducing sugars, and increased phenol levels. Joint treatment of SAR and root-knot inoculation resulted in an additive effect, especially with regard to the levels of non-reducing sugars and soluble phenols. An inverse correlation was observed between sugar content and the levels of soluble phenols, which is presumably the consequence of diversion of precursors of sugar metabolism to the synthesis of secondary metabolites such as phenols. The physiological relationship between the response of okra plants to SAR and RKN in combination seems to be additive, as evidenced in Tables II-V.

All the treatments, in particular the combined treatments of SAR and RKN, suppressed the yield of okra, presumably due to a reduction of photosynthesis.

SAR treatment in combination with RKN inoculation suppressed the reproduction of *M. incognita.* The reproductive stage of okra and *M. incognita* more or less coincided, leading to a maximum burden on the host plant for nutrients and, therefore, a considerable shortage of nutrients for sedentary female nematodes, which would in turn limit fecundity. Oteifa (1953) and Khan and Khan (1994b) demonstrated that a deficiency of nutrients inhibits the reproductive capacity of *M. incognita*.

The present study has demonstrated the possibility of an additive interaction between acid rain (pH 4.0) and RKN infection on okra. Also, it seems that SAR exposure induces physiological changes that could alter the pathogenic response of the plants to *M. incognita*. The reproductive capacity of *M. incognita* in particular is affected by SAR.

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