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Interactions of *Meloidogyne incognita, Xanthomonas campestris*, and *Rhizobium* sp. in the disease complex of chickpea

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Abstract: The effects of *Meloidogyne incognita*, *Xanthomonas campestris*, and *Rhizobium* sp., alone or in combination, on the disease complex in chickpea were examined. Individual inoculation with *M. incognita* and *X. campestris* caused significant reductions in plant growth, while inoculation with *Rhizobium* sp. resulted in a significant increase in plant growth. Inoculation with *M. incognita* and *X. campestris* together caused a greater reduction in plant growth than the damage caused by each of them alone. Application of *M. incognita* or *X. campestris* prior to *Rhizobium* sp. caused a greater reduction in plant growth than *Rhizobium* sp. applied prior to *M. incognita* or *X. campestris*. Application of *M. incognita* prior to *X. campestris* resulted in the maximum reduction in plant growth. Use of *M. incognita* or *X. campestris* with *Rhizobium* sp. reduced root nodulation. Application of *Rhizobium* sp. prior to pathogens resulted in greater nodulation than *Rhizobium* sp. applied simultaneously with pathogens. *Rhizobium* sp. and *X. campestris* had adverse effects on galling and nematode multiplication. Maximum reduction in galling and nematode multiplication was observed when *Rhizobium* sp. and *X. campestris* were applied together prior to *M. incognita*.

Key words: Chickpea, *Meloidogyne*, *Xanthomonas*, *Rhizobium* sp., disease complex, interactions

1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important pulse crops in India, ranking fourth among grain crops in acreage and production. It occupies about 6.67×10^6 ha, producing 5.3×10^6 t annually in India (Singh 2012).

A large number of nematodes are known to cause diseases in chickpea, but *Meloidogyne incognita* (order Tylenchida, family Heteroderidae) is of significant importance in India (Gill 1989). One of the important bacterial diseases of chickpea is the bacterial blight and damping off caused by *Xanthomonas campestris* pv. *cassiae* (Rangaswami and Mahadevan 2006).

During the course of a survey for plant parasitic nematodes and bacteria (from both the rhizosphere and rhizoplane) in the Aligarh district of Uttar Pradesh, we observed the frequent and simultaneous occurrence of *M. incognita* (Kofoid and White) Chitwood and *X. campestris* (Pammel 1895) Dowson 1939 in the root and soil samples collected from chickpea fields. When both pathogens were present together plants were severely galled and wilted, as both the inhibition and acceleration of many plant diseases are influenced by associated organisms. The root nodule bacterium *Rhizobium* sp. was also associated in chickpea under field conditions. Therefore, we decided to study the effect of interactions among *M. incognita*, *X. campestris*, and *Rhizobium* sp. on chickpea growth and disease development.

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2. Materials and methods

Meloidogyne incognita and *Xanthomonas campestris* were selected as the test pathogens and chickpea variety Pusa 1058 as a test plant.

2.1. Preparation and sterilization of soil mixture

Soil, river sand, and organic manure were mixed in a ratio of 3:1:1, and 15-cm clay pots were filled with 1 kg of soil per pot. To each pot, 50 mL of water was added to wet the soil before transfer to an autoclave for sterilization at 137.89 kPa for 20 min. Sterilized pots were allowed to cool down at room temperature before use.

2.2. Raising and maintenance of test plant

Chickpea seeds (variety Pusa 1058) were surface-sterilized with 0.1% sodium hypochlorite solution for 2 min and washed 3 times in sterilized water before sowing. In each pot 5 seeds were sown, and thinning was done after germination to maintain 1 plant per pot. Watering was done whenever required. One-week-old, well-established healthy seedlings were used for the experiment.

2.3. Preparation of nematode inoculums

Large numbers of egg masses from heavily infected eggplant (*Solanum melongena* L.) roots were hand-picked with the help of sterilized forceps from the previously maintained pure *Meloidogyne incognita* culture. The egg masses were washed with distilled water and placed in a small sieve containing crossed layers of tissue paper. The sieve was placed in a petri plate containing water. A number of these assemblies were kept in order to obtain the required number of second-stage juveniles for inoculation. The hatched second-stage juveniles were collected from the petri plates every 24 h, fresh water was added, and the process was repeated. For counting nematode juveniles, an average of 5 counts were made to determine the density of nematodes in the suspension. Into each plant, 1000 freshly hatched second-stage juveniles were inoculated.

2.4. Preparation of bacterial inoculums

Nutrient agar plates were prepared by pouring sterilized nutrient agar (beef extract, 1 g; yeast extract, 2 g; peptone, 5 g; NaCl, 5 g; agar, 15 g; distilled water, 1 L) into petri plates and then incubating overnight at 30 °C to check for sterility and remove excess moisture. The nutrient agar plates were streaked with a pure colony of *Xanthomonas campestris* and incubated at 32 ± 1 °C for 24 h. A single colony from this freshly cultured subplate was inoculated into each nutrient broth flask and incubated at 32 ± 1 °C for 72 h. One milliliter contains about 1.2×10^5 colony-forming units (CFU) mL^{-1} of *X. campestris* bacteria. For the calculation of CFU mL^{-1}, serially diluted suspensions in steps of 10% were plated on nutrient agar plates and incubated at 37 °C for 24 h. Colonies on petri plates falling within the 30–300 range were selected and multiplied with a dilution factor to obtain the bacterial colony number (Sharma 2001). Into each pot, 10 mL of this suspension was inoculated around the chickpea seedling.

2.5. *Rhizobium* inoculum

To prepare the *Rhizobium* inoculum, 100 g of commercial *Rhizobium* sp. culture was dissolved in 1 L of distilled water. Ten milliliters of this suspension containing 1 g of inoculum was inoculated per plant.

2.6. Inoculation technique

There were 2 inoculations performed in order to study the effect of pre- and postestablishment of the pathogens and *Rhizobium* sp. on disease development. The second inoculation was done 15 days after the first. To inoculate the seedlings, feeder roots were carefully exposed by removing the top soil layer around the roots. The inoculum was uniformly poured around exposed roots using a sterilized pipette. After inoculation, the soil was replaced. The inoculations were performed as shown in the Table. Each set was replicated 5 times and watered as needed. The experiment was terminated 90 days after the first inoculation.

2.7. Recording of observations

Plants were uprooted 90 days after the first inoculation. Root systems were gently washed taking the utmost care to avoid losses and injuries during the operation. Plant length in millimeters was recorded from the top of the first leaf to the base of the root. Excess water was removed from the plant roots by placing them between 2 folds of blotting sheets. Shoots and roots were kept in envelopes in an oven at 60 °C for 2–3 days for drying, and then dry weight in grams was recorded. The number of nodules and galls per root system was estimated. To extract nematodes from the soil, a 250-g subsample from the well-mixed soil of each treatment was processed by Cobb's sieving and decanted with a Baermann funnel (Southey 1986). Nematode suspensions were collected after 24 h, numbers of nematodes were counted in a counting dish, and 5 replicates of a 1-mL suspension were taken from each sample. The means of 5 such counts were obtained, and the population of nematodes per kilogram of soil was calculated. In order to estimate juveniles, eggs, and females inside the roots, a 1-g root sample was taken from the homogeneous mixture of root and macerated for 30–40 s in a Waring blender; counting was done using the suspensions obtained. A scale of 0–5 was used as the bacterial blight index, where 0 = no disease and 5 = severe bacterial blight.

2.8. Statistical analysis

Data were subjected to analysis of variance. Tukey's test was used to distinguish differences between treatments. All analyses were performed with StatView 5.0 (SAS Institute, Cary, NC, USA).

3. Results

Inoculation with *Rhizobium* sp. caused an increase in plant growth based on plant dry weight over the uninoculated control, while inoculation with *M. incognita* and *X. campestris* caused significant reductions in plant growth (Table). Inoculation with *M. incognita* caused a greater reduction in plant growth than inoculation with *X. campestris*. Inoculation with *M. incognita* + *X. campestris* together caused a greater reduction in plant growth than the damage caused by either of them alone. Inoculation with *Rhizobium* sp. together with *M. incognita* or with *X. campestris* caused a reduction in plant growth similar to inoculation with *M. incognita* or *X. campestris* alone. Inoculation with *Rhizobium* sp. prior to *M. incognita* or *X. campestris* reduced damage caused by pathogens compared to plants in which pathogens were introduced prior to *Rhizobium* sp. Inoculation with *M. incognita* prior to *Rhizobium* sp. caused a greater reduction in plant growth than *Rhizobium* sp. introduced prior to *M. incognita*. Inoculation with *M. incognita* prior to *X. campestris* caused a greater reduction in plant growth than

Table. Interactions of *Meloidogyne incognita*, *Xanthomonas campestris*, and *Rhizobium* sp. in chickpea.

Treatments		Plant length (mm)	Plant fresh weight (g)	Plant dry weight (g)	No. of nodules root per system	No. of galls per root system	Nematode population	Bacterial blight index
Control	Individual inoculation	820.8b	29.03b	7.85b	3h	-	-	-
Rh[a]		920.3a	33.63a	9.08a	45a	-	-	-
Mi[b]		590.4f	20.32g	5.80ef	2h	171a	27360a	-
Xc[c]		630.1e	22.98ef	6.45cd	2 h	-	-	3
Rh + [d]Mi	Combined inoculation of 2 organisms	660.4de	23.06def	6.07de	23de	143c	22980c	-
Rh + Xc		680.6cd	24.28cde	6.52cd	27c	-	-	3
Mi + Xc		460.7k	15.36hij	4.42jk	1h	122e	19540e	5
Rh→[e]Mi	One organism first and another later	690.7cd	23.95cde	6.59cd	36b	129de	20530d	-
Rh→Xc		710.2c	25.06c	7.06c	39b	-	-	2
Mi→Rh		630.7e	21.76fg	5.60efg	19ef	158b	25170b	-
Mi→Xc		420.8l	14.29j	3.90k	2h	140cd	22350c	5
Xc→Mi		510.7ghi	16.38hi	4.78hij	3h	106f	16790f	5
Xc→Rh		660.4de	24.72cd	6.06de	21de	-	-	4
Rh→Mi + Xc	One organism first and 2 later	530.7gh	16.54h	5.32fgh	25cd	87gh	14160i	5
Mi→Rh + Xc		480.2ijk	15.59hij	4.67ij	20ef	98fg	15280h	5
Xc→Mi + Rh		520.6gh	16.68h	5.18fghi	23de	81hi	12760k	5
Rh + Mi→Xc	Two organisms first and another later	520.6gh	16.54h	5.07ghi	20ef	104f	16610f	5
Mi + Xc→Rh		470.9jk	14.83ij	4.77hij	16fg	102f	15980g	5
Rh + Xc→Mi		540.8g	16.16hi	5.26fghi	22de	74i	11760l	5
Rh + Mi + Xc	All 3 together	500.5hij	16.08hi	4.98ghij	15fg	88gh	13740j	5

[a]Rh = *Rhizobium* sp.; [b]Mi = *Meloidogyne incognita*; [c]Xc = *Xanthomonas campestris*. +[d] = simultaneous inoculation; →[e] = inoculation 15 days after first inoculation. Values in a column followed by the same letter are not significantly different ($P \leq 0.05$).

X. campestris introduced prior to *M. incognita*. Inoculation with *M. incognita* prior to *Rhizobium* sp. together with *X. campestris* caused a greater reduction in plant growth than *X. campestris* introduced prior to *M. incognita* + *Rhizobium* sp. Inoculation with *M. incognita* + *X. campestris* prior to *Rhizobium* sp. caused a reduction in plant growth similar to inoculation with *M. incognita* prior to *Rhizobium* sp. + *X. campestris*. Simultaneous inoculation with *Rhizobium* sp., *M. incognita*, and *X. campestris* caused a reduction in plant growth similar to inoculation with *Rhizobium* sp. + *M. incognita* followed by *X. campestris* 15 days later (Table).

Nodulation was high in plants inoculated with *Rhizobium* sp. alone (Table). Only a few nodules were observed in plants not inoculated with *Rhizobium* sp. Inoculation of *M. incognita* or *X. campestris* with *Rhizobium* sp. reduced root nodulation as compared to plants inoculated with *Rhizobium* sp. alone. Inoculation with *Rhizobium* sp. prior to pathogens resulted in greater nodulation than inoculation with *Rhizobium* sp. simultaneously with pathogens. Nodulation was found less often in plants inoculated with *Rhizobium* sp. + both pathogens than in plants inoculated with *Rhizobium* sp. and a single pathogen. Among plants inoculated with *Rhizobium* sp., the least nodulation was observed when both pathogens + *Rhizobium* sp. were introduced simultaneously or *M. incognita* + *X. campestris* was introduced prior to *Rhizobium* sp. (Table).

Rhizobium sp. and *X. campestris* had adverse effects on galling and nematode multiplication. *X. campestris* had a greater adverse effect on galling and nematode multiplication than *Rhizobium* sp. Inoculation with *Rhizobium* sp. together with *X. campestris* caused a greater reduction in galling and nematode multiplication than inoculation with either of them alone. Maximum reduction in galling and nematode multiplication was observed when *Rhizobium* sp. + *X. campestris* was introduced prior to *M. incognita* (Table). The bacterial blight index was 3 when *X. campestris* was introduced alone or *X. campestris* was introduced with *Rhizobium* sp. The index was 2 when *Rhizobium* sp. was introduced prior to *X. campestris*, and the bacterial blight index was 4 when *X. campestris* was introduced prior to *Rhizobium* sp. For treatments in which *X. campestris* was introduced in combination with *M. incognita* and *Rhizobium* sp., the indices were 5 (Table).

4. Discussion

Interaction of the root-knot nematode in *M. incognita* with *X. campestris* in chickpea causes a disease complex under field conditions. Inoculation with these pathogens alone caused a significant reduction in plant growth over the control. The root-knot nematode has evolved strategies to induce feeding cell formation in many plants in addition to chickpea, probably by manipulating the fundamental elements of plant cell development (Caillaud et al. 2008); this has caused significant yield loss. Moreover, *X. campestris* can severely devitalize plants through defoliation, and it reduces yield and quality. This disease is characterized by water-soaked lesions of young root tissue, which turn dark brown. Water-soaked lesions also appear on leaf surfaces and later turn into dark brown lesions with chlorotic holes. Over time the infected tissues appear to lose their structural integrity and disintegrate, taking on a soft-rot appearance. Inoculation with combinations of these pathogens caused greater damage in chickpea than inoculation with individual pathogens. Interactions between these pathogens may have both direct and indirect effects on disease severity. Direct effects include the physical interaction of pathogens in the rhizosphere and occupancy of the same infection site inside the root. Direct interaction of pathogens inside host plants at the same infection site generally had an antagonistic effect on pathogen multiplication. Indirect effects of the interactions via plant response, such as breaking of disease resistance and modification of the host substrate, had synergistic effects on disease severity. Plant parasitic nematodes cause physical damage that may allow secondary infection through other pathogens (Pitcher 1963, 1965; Sitaramaiah and Pathak 1993). Endoparasitic nematodes such as *Meloidogyne* spp. injure roots, allowing bacteria to become established (Stewart and Schindler 1956; Siddiqui et al. 2012).

Most pathogenic bacteria depend mainly on wounds as an infection court (Goodman et al. 1967), and these wounds are created by nematodes feedings on roots (Sitaramaiah and Sinha 1984a, 1984b). Pitcher (1965) noted that wounds created by nematodes apparently favor bacteria more than fungi, because bacteria are less adapted for penetrating the host epidermis. Disease symptoms similar to those occurring in nematode–bacteria wilt interactions were simulated by substituting mechanical injury for nematode feeding (Lucas et al. 1955; Libman et al. 1964). When chickpea roots were mechanically injured by needle in laboratory tests and plants were inoculated with *X. campestris*, the plants exhibited disease symptoms similar to those occurring in nematode–bacterial wilt interactions. Wounds created by nematodes leak nutrients, allowing bacteria to multiply in lesions and the rhizosphere (Kurppa and Vrain 1985). It was observed in the present study that rhizosphere soil had higher bacteria populations than nonrhizosphere soil. Moreover, the root-knot nematode induces physiological and/or biochemical changes in the hosts.

Inoculation with *Rhizobium* sp. improved plant growth over the uninoculated control by increasing the nitrogen status of the soil. In cases where *Rhizobium* sp.

was used with pathogens, it also improved plant growth by producing antibiotics against the pathogens. *Rhizobium* sp. produces antipathogenic substances (Marx 1969; Drapeau et al. 1973), and plants inoculated with *Rhizobium* sp. suffered less damage by pathogens than uninoculated plants (Bopaiah et al. 1976; Tu 1978, 1980). Prior establishment of *Rhizobium* sp. produced more antibiotics and improved plant growth more than establishment of *Rhizobium* sp. together with or after the pathogens. Similarly, *X. campestris* has adverse effects on nematode multiplication. The adverse effect of *X. campestris* on nematodes may be due to competition for the same host substrate. The unfavorable effect of bacteria on nematodes may be due to the destruction of feeding sites, which reduces the available nutrition for nematodes; this was observed in tobacco plants in Granville wilt and root-knot nematode interaction (Lucas et al. 1955). Inoculations of root-knot nematodes alone produce more galls and egg masses than when *X. campestris* is present. It may be that bacteria induce changes in the root system that are not favorable for nematodes (Hazarika 2003; Hussain and Bora 2009). Generally, bacteria adversely affect nematode multiplication; however, inoculation with nematodes and plant-pathogenic bacteria increased disease severity by predisposing plants to pathogenic bacteria. When inoculation with bacteria occurred prior to inoculation with nematodes, damage to plants was less severe than with simultaneous inoculations. This may be due to the production of toxins by bacteria, which adversely affects the nematodes (Pitcher 1963). Perhaps bacteria could not infect the roots effectively without the infection courts provided by the nematodes. Inoculation with *Rhizobium* sp. and *X. campestris* together caused a greater adverse effect on nematode galling and its multiplication than either inoculum used alone. This may be due to the added adverse effects of *Rhizobium* sp. and *X. campestris* on nematode multiplication.

Both nematodes and bacterial pathogens had adverse effects on nodulation caused by *Rhizobium* sp. Nematodes and bacterial pathogens together have a greater negative effect on nodulation than any of them used alone (Mani and Sethi 1987). Similarly, inoculation with a pathogen prior to *Rhizobium* sp. produced a greater adverse effect on nodulation than inoculation with *Rhizobium* sp. prior to the pathogens. This is possible because prior inoculation of pathogens creates conditions unfavorable for *Rhizobium* sp. establishment. Similarly, when *Rhizobium* sp. was introduced prior to pathogens, the antipathogenic substances produced by *Rhizobium* sp. adversely affected pathogen establishment.

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