ANATOMICAL CHANGES INDUCED BY TWO SOIL-BORNE PATHOGENS (*PLASMODIOPHORA BRASSICAE* AND *MELOIDOGYNE JAVANICA*) IN CABBAGE

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Summary. Stunted growth of large patches of cabbage cv. Lupini, associated with severe soil infestations by the root-knot nematode *Meloidogyne javanica* and the protist *Plasmodiophora brassicae*, the casual agent of clubroot disease, was observed in several fields at Castellaneta, province of Taranto, in southern Italy. The host-parasite responses of cabbage roots to parasitism by the two soil-borne pathogens was studied and compared. In roots infected by *P. brassicae*, the plasmodia were present in cortex and pericycle cells, causing hypertrophy and hyperplasia, and developed into resting spores within host tissues. Parasitism of *M. javanica* was characterized by the establishment of distinct permanent feeding sites with giant cells in the cortex, endodermis and vascular parenchyma, which limit water and nutrient translocation.

Keywords: *Brassica oleracea* var. *capitata,* clubroot, histopathology, host-parasite relationships, Italy, root-knot nematodes.

Cabbage (*Brassica oleracea* var. *capitata*) is commercially cultivated for its large and leafy head that is rich in vitamin C. It is regarded as the most significant member of the Brassicaceae (Cruciferae) family and has remained one of the world's leading vegetable crops. Cabbage is also an economically important vegetable crop in several Mediterranean countries, including Republic of Serbia, Italy, Portugal, Spain and Greece (FAOSTAT, 2010). In Italy, cabbage is widely cultivated (about 17,000 ha) especially in southern regions. In the Mediterranean basin, under field conditions, cabbage is usually transplanted in late summer and harvested in early spring, and is exposed to infection by several diseases, particularly those caused by soil-borne pathogens. Several diseases have been reported damaging cabbage worldwide. While some of them may simply cause minor spotting, others can be devastating for the crop. Among them, clubroot, *Plasmodiophora brassicae* Woronin, black rot, *Xanthomonas campestris* (Pammel) Dowson, black leg, *Leptosphaeria maculans* (Desmaz.) Ces. *et* De Not., imperfect state *Phoma lingam* (Tode:Fr.) Desmaz., downy mildew, *Peronospora parasitica* Pers. ex Fr., yellows, *Fusarium oxysporum* f. sp. *conglutinans* (Wollenweb.) Snyder *et* Hans., and plant-parasitic nematodes, particularly root-knot nematodes, *Meloidogyne* spp., are considered major diseases (Rimmer *et al*., 2007).

Plasmodiophora brassicae is an obligate parasite that causes clubroot in many Brassicaceae such as canola (oilseed rape), broccoli, brussels sprouts, cabbage, kale, kohlrabi, radish, rutabaga, cauliflower, turnip and black mustard (Buchwaldt and Rimmer, 2007). The life cycle of the parasite consists of two main phases, the first occurring in root hairs and the second in cells of the root cortex and stele leading to gall formation and production of haploid resting spores. Root galls (clubs), the most characteristic symptom of *P. brassicae* on infected plants, inhibit nutrient and water transport, stunt the growth of the plant and induce premature wilting, which usually results in severe yield losses (Ingram and Tommerup, 1972; Voorrips, 1995). However, similar symptoms are also induced by other root pathogens, such as the crown gall bacterium *Agrobacterium tumefaciens* (Smith *et* Townsend) Conn, wart fungi, *Physoderma* sp., *Spongospora* sp., and root-knot nematodes. The root-knot nematode species most damaging to cabbage are *M. arenaria* (Neal) Chitw., *M. artiellia* Franklin, *M. hapla* Chitw., *M. incognita* (Kofoid *et* White) Chitw., *M. javanica* (Treub) Chitw. and *M. enterolobii* Yan *et* Eisenback (= *M. mayaguensis* Rammah *et* Hirschmann) (Potter and Olthof, 1993; Abrantes *et al*., 1994; Sikora and Fernández, 2005). *Meloidogyne* spp. prevailing in the Mediterranean basin, such as *M. arenaria*, *M. incognita* and *M. javanica* are warm climate species. However, the low soil temperatures reached in the Mediterranean basin in fall and winter are not suitable for infection and development of these *Meloidogyne* species (Moens *et al*., 2009) and, therefore, the incidence of root-knot nematodes in cabbage in this region is usually low. In contrast, cool, wet and acidic soils provide the most favourable environment for *P. brassicae*. Resting spores of this pathogen germinate in the presence of host roots when temperatures are at least 16 ºC, while host infection is favoured by temperatures above 18 ºC and disease severity increases with rising temperatures up to 26 ºC (Buczacki *et al.*, 1978).

In spite of the severe damage that both pathogens can cause in susceptible crops, and although a vast liter-

ature is available on different aspects of these two pathogens, there is a lack of information concerning the host-parasite relationships of clubroot and root-knot nematode diseases in cabbage. During early October 2007, severe feeder root infections (club-like swellings and root-knots) of cabbage cv. Lupini were found in fields at Castellaneta, province of Taranto, in southern Italy. Hence, the main goals of this study were to determine the causal agents of the disease and to investigate the host-parasite interactions between cabbage roots, *P. brassicae* and *M. javanica*.

MATERIALS AND METHODS

Roots and rhizosphere and bulk soil samples were taken from the stunted patches in a cabbage field at Castellaneta.

The identification of the clubroot pathogen was based on visible symptoms and microscopic examinations according to available descriptions (Ingram and Tommerup, 1972; Buczacki, 1979; Mithen and Magrath, 1992).

The identification of the root-knot nematode species was based on: 1) morphological and morphometric observations of 25 second-stage juveniles (J2) and stylet knobs of the males, extracted from soil by the Coolen (1979) method and prepared according to Seinhorst (1962); 2) morphology of 20 perineal patterns of females, prepared as described in Hartman and Sasser (1985), and excretory pore position/stylet length ratio of adult females; and 3) esterase and malate dehydrogenase phenotypes of protein extracts from five young egg-laying females (Esbenshade and Triantaphyllou, 1985). A reference *M. javaniva* isolate, from Córdoba, Spain, was included in each gel.

Nematode population densities in soil and roots were assessed from the stunted patches. Nematodes from soil were extracted from a 100-cm^3 subsample using the centrifugal-flotation method (Coolen, 1979). Nematodes from galled roots were extracted from a 5-g root subsample washed free of soil and blended in a 0.5% NaOCl solution for 4 min (Hussey and Barker, 1973).

Clubbed and galled roots, from naturally infected cabbage plants in the same field, were selected for histopathological studies. Root tissues were gently washed free of adhering soil and debris and individual galls were selected along with healthy roots. These root segments were fixed in formaldehyde chromo-acetic solution for 48 h, dehydrated in a tertiary butyl alcohol series (40-70-85-90-100 %), embedded in 58 °C-melting point paraffin and sectioned with a rotary microtome. Sections of 10-12 µm thickness were placed on glass slides, stained with safranin and fast-green, mounted permanently in 40% xylene solution of a polymethacrylic ester (Synocril 9122X, Cray Valley Products, NJ, USA), examined microscopically and photographed (Johansen, 1940).

RESULTS AND DISCUSSION

Cabbage plants infected by clubroot and root-knot nematodes within the patches were severely stunted and presented heavily deformed and damaged root systems (Fig. 1).

The causal agent of the clubroot was identified as *Plasmodiophora brassicae*. Symptoms caused by *P. brassicae* included abnormal enlargement (clubs) of the roots with severe swelling of main and lateral roots and below ground stems. In severely affected plants, clubs coalesced and covered most of the root system (Fig. 1B). As a consequence, plants showed reduced growth and stunting (Fig. 1A). Based on club size and intensity of root malformation, cabbage cv. Lupini should be considered highly susceptible to *P. brassicae* (Buczacki *et al.*, 1975).

Morphometric observations of J2s, males, perineal patterns and excretory pore position/stylet length ratios of the females were in agreement with those typical of *M. javanica* (Orton Williams, 1972). The enzymatic studies revealed the presence of a species-specific phenotype (J3) and a non-specific dehydrogenase (N1) phenotype, both of which have been associated with *M. javanica* populations (Esbenshade and Triantaphyllou, 1985). These bands were similar to those of the reference population of *M. javanica* from Córdoba, Spain.

The histological sections showed that *P. brassicae* infected the root cortex, reached the vascular tissue and promoted an abnormal increase in cabbage cell division (hyperplasia) and enlargement (hypertrophy) that resulted in extensive disorganization of tissue structure (Fig. 1C, D). Infected cells occurred in clusters of variable size throughout diseased tissues surrounded by uninfected healthy looking cells (Fig. 1D). Multinucleate, intra-cellular plasmodia were easily observed within the clubs in the cortex and pericycle cells. Immature plasmodia were also present in the affected tissue. Plasmodia ultimately develop into resting spores (Fig. 1C, D). These histological changes and pathogen structures are similar to those described previously for the second infection cycle of *P. brassicae* in susceptible hosts (Ingram and Tommerup, 1972; Mithen and Magrath, 1992; Kobelt *et al.*, 2000; Ando *et al.*, 2006).

Root galls induced by *M. javanica* in cabbage occurred on both the main and lateral roots and varied in size and shape. Large root-knot nematode galls containing one or more females, males and eggs, were detected (Fig. 1F, H, I) and nematode population density ranged from 4,100 to 6,800 eggs and J2/g of galled fresh roots. Generally, large, spherical regular galls were present on root tips, and several were also present along the root axes. Galls occurred either singly or in clusters, which could encircle the entire root and resulted in noticeable distortions of the entire root morphology. In this latter case, the root diameter was from four to eight times that of uninfected roots. Occasionally, an egg mass was found inside the cortical root tissues, but the majority of the egg masses were protruding from the root surface

(Fig. 1I). Histological sections of healthy and *M. javanica*-infected cabbage roots confirmed that the nematode successfully established permanent feeding sites, which caused cellular alterations in the root cortex, endodermis, pericycle, and vessels due to nematode-induced cellular expansion of giant cells and to the expanding bodies of the nematode females (Fig. 1H, I). In most cases, the permanent feeding sites induced by the nematode adjacent to the vascular tissues consisted of groups of 2 to 4 large, multinucleate giant cells, but 3 to 6 giant cells per feeding site were also commonly observed (Fig. 1H, I). Often, these feeding sites caused severe distortion and crushing of xylem tissues. The giant cells showed characteristically dense cytoplasm and 8 to 24 hypertrophied nuclei and nucleoli (Fig. 1I). Additionally, hyperplasia of tissues adjacent to the giant cells contributed to root tissue expansion, leading to the formation of root galls.

In this research, the large numbers of plasmodia containing resting spores of *P. brassicae*, together with the large numbers of egg masses, J2, and eggs of *M. javanica* recovered from infected cabbage roots or rhizosphere soil, indicate a successful host-parasite relationship between the two pathogens and cabbage cv. Lupini, and

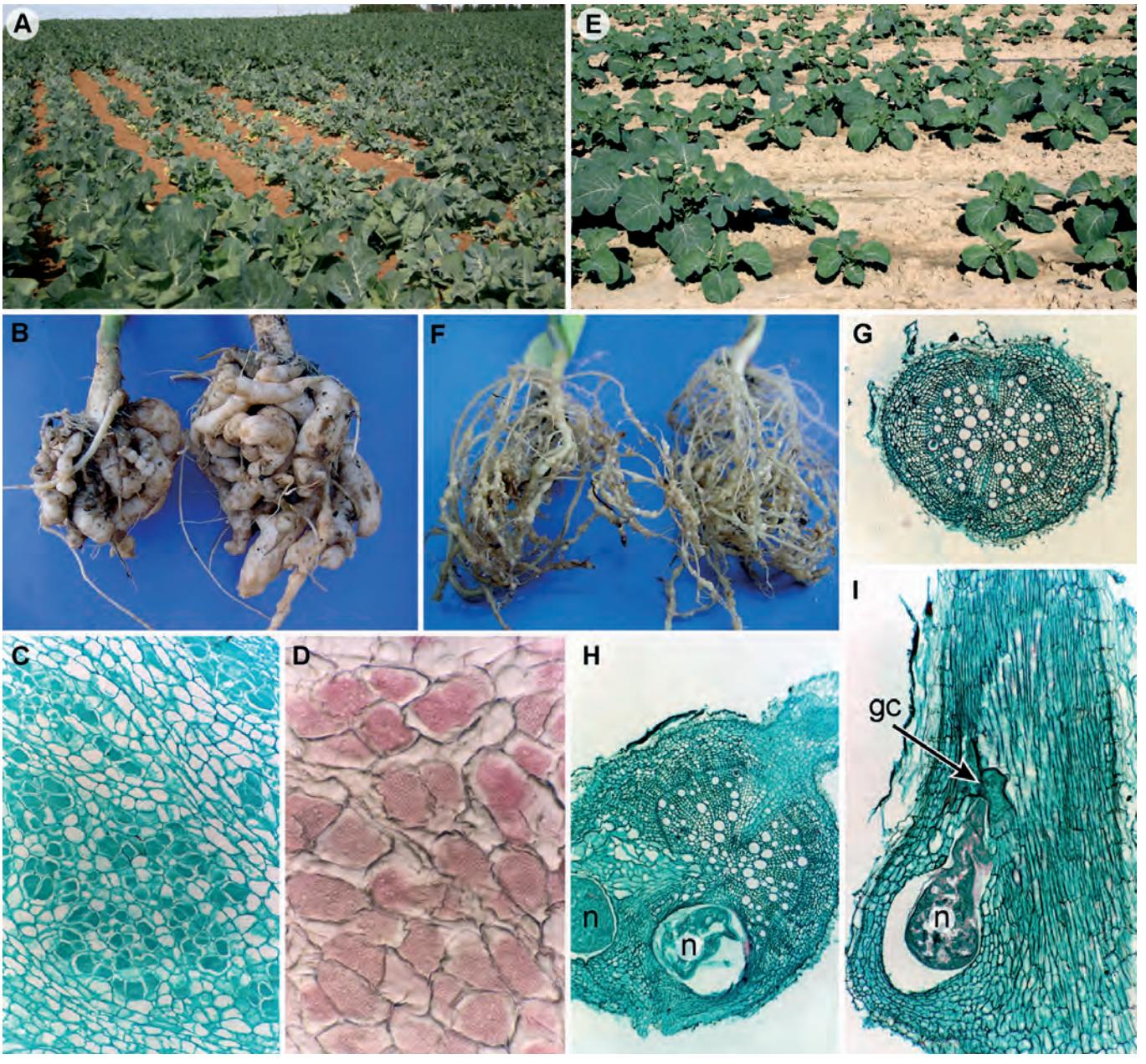

Fig. 1. Cabbage cv. Lupini infected by *Plasmodiophora brassicae* (A-D) and by *Meloidogyne javanica* (E-I) in a field in southern Italy. A, E) Field showing patches with stunted plants. B) Root systems showing clubroot symptoms with extensive galling. C-D) Cross sections of roots showing the plasmodia in hypertrophied cortical cells. F) Root system severely infected by *M. javanica* showing large and numerous galls. G) Cross section of a healthy cabbage root. H, I) Cross and longitudinal sections of a root showing the nematode (n) feeding on multinucleate giant cells (gc) with hypertrophied nuclei.

qualify the latter as a satisfactory or excellent host for *P. brassicae* (Buczacki *et al.*, 1975) and *M. javanica* (Hussey, 1985). Moreover, our results indicate that sowing susceptible cabbage in *P. brassicae* or *M. javanica* infested soils would lead to a dramatic increase in soil inoculum through release of *P. brassicae* resting spores or *M. javanica* egg masses from infected root tissues that would make these soils unfit for cultivation of susceptible crucifers. In the present study, no concomitant infections were observed. In addition, although glucosinolates stored in the vacuoles of cabbage cells have shown nematicidal activity against some plant-parasitic nematodes (Potter *et al.*, 1999), release of these toxic compounds requires disruption of cells. *Meloidogyne* spp. move intercellularly and cause minimal cell damage while migrating through root tip regions (where cells are young and not vacuolated) to the vascular cylinder, where they settle and grow (Wyss *et al.*, 1992). These findings may be responsible for the lack of nematicidal activity of cabbage against *Meloidogyne* spp. In this context, glucosinolate content has also been found to play a role in the development of symptoms induced by *P. brassicae*, it being suggested that host genotypes with lower concentrations of indole glucosinolates might show reduced clubroot symptoms. However, glucosinolate content can increase dramatically in cabbages infected by *P. brassicae* and, therefore, interact with infections by root-knot nematodes (Butcher *et al.*, 1974; Ludwig-Müller *et al.*, 1997).

The present findings on the host-parasite relationships of *P. brassicae* and *M. javanica* in cabbage indicate that, under field conditions in the Mediterranean area with warm autumns, these soil-borne pathogens can be highly virulent to this crop and cause severe losses and increase inoculum in soil. The use of resistant cabbage cultivars would be the most practical control measure for both pathogens. Unfortunately, to our knowledge, there are no cabbage cultivars possessing combined resistance to both pathogens. Therefore, if pathogen-free soils are not available, some control practices, such as crop rotation, nematicides or fungicides, soil solarization, organic amendments, biological control, or nematicidal plants, should be used to reduce infestation level to or below the tolerance limit of the target pathogen, particularly under warm environmental conditions suitable for root-knot nematode development and reproduction. However, this requires that growers become aware of the impact of these pathogens on cabbage and increase their reliance on soil sampling to assess the risk of pathogen damage and to select proper cropping sequences and possible control strategies.

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