

**Orijinal araştırma (Original article)**

**Histopathology of *Brassica oleracea* var. *capitata* subvar. *alba* infected with *Heterodera cruciferae* Franklin, 1945 (Tylenchida: Heteroderidae)**

*Heterodera cruciferae* Franklin, 1945 (Tylenchida: Heteroderidae) ile bulaşık *Brassica oleracea* var. *capitata* subvar. *alba*'nın histopatolojisi

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**Summary**

Anatomical changes induced by the cabbage cyst nematode (*Heterodera cruciferae*) have been insufficiently characterized. Here these changes were described in the root tissues of white head cabbage variety (Yalova F1) commonly grown in the Black Sea region of Turkey, where cabbage-growing areas are heavily infested. In glasshouse experiments conducted at 20 degrees C, susceptible white head cabbage seedlings were inoculated with 0 (untreated control) or 1000 juveniles/300 ml soil. Three, 6, 12, 24, 48, 72 h and 30 days after inoculations, two plants from each treatment were removed, embedded in paraffin by using microwave technique and then examined by photomicrography. Second-stage passed through the vascular system after root penetration and they started to feed as sedentary. In cross section of the roots, large cells in the cortex of infected plants were filled with moderately dense cytoplasm and the walls were heavily stained and ruptured. In longitudinal section, internal walls were perforated. Syncytia that had different degrees of vacuolization, and syncytial nuclei were hypertrophied and deeply indented. Contained conspicuous nucleoli were noticeable 24 h after inoculation. Syncytia originating from endodermal cells possessed ruptured walls around the feeding site of the developing juvenile. White females were observed on the roots 30 days after inoculation, a time at which plant height was reduced and root proliferation increased. Concurrently, above- and below-ground symptoms were also observed.

**Key words:** Histopathology, *Brassica*, cabbage cyst nematode, *Heterodera cruciferae*

**Özet**

*Heterodera cruciferae*'nin dokularda oluşturduğu değişiklikler ayrıntılı olarak araştırılmadığından, bu çalışmada Karadeniz Bölgesinde en yaygın olarak yetiştirilen ve doğal olarak yaygın bir şekilde bu nematodla bulaşık olan beyaz baş lahana (Yalova F1) çeşidindeki histopatolojisi araştırılmıştır. Hassas beyaz baş lahana fideleri, 20°C serada yürütülen denemelerde, 0 (kontrol) veya 1000 juvenil/300 ml toprak olacak şekilde bulaştırılmıştır. Bulaştırmalardan 6, 12, 24, 48, 72 saat ve 30 gün sonra, her uygulamadan 2 bitki alınarak, mikrodalga yöntemiyle paraffin içine gömülerek, mikroskop altında incelenmiş ve fotoğraflanmıştır. Bu incelemelerde, köke giren ikinci dönem larvaların vascular sisteme doğru hareket ederek kalıcı forma geçip hemen beslenmeye başladıkları saptanmıştır. Nematodla bulaşık bitkilerin kortekslerinin enine kesitlerinde, hücrelerdeki büyüme, stoplazmadaki yoğunluk ve hücre duvarlarındaki yırtık ve delikler kolayca görülmektedir. Boyuna kesitte de internal duvarlardaki yırtıklar belirgindir. Nematodla bulaştırdıktan 24 saat sonra oluşan syncytianın, farklı seviyelerde vakuolizasyonu görülmektedir ve bu alandaki hücreler hipertroftiktir. Yırtılan hücrelerin birleşmesiyle de endodermal hücrelerdeki syncytia, gelişmekte olan juvenillere besin alanı teşkil etmektedir. Bulaştırmalardan 30 gün sonra beyaz dişiler elde edilmiştir ki aynı zamanda bitki ağırlığı azalmış ve köklerde proliferasyon (yan kök sayısında artış) saptanmıştır. Aynı zamanda, toprak üstü ve altı belirtiler de kaydedilmiştir.

**Anahtar sözcükler:** Histopatoloji, *Brassica*, lahana kist nematodu, *Heterodera cruciferae*

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## Introduction

*Heterodera cruciferae* Franklin, 1945 (Tylenchida: Heteroderidae), the cabbage cyst nematode, is widely distributed throughout the world, especially in cabbage-growing areas (Stone & Rowe, 1976; Sturhan & Liskova, 2004; Jabbari & Niknam, 2008). The nematode infects only crucifers and persists in the soil in the absence of a host crop; and two to three generations may be completed in one year. In severe infestations, long crop rotations of more than 6 years are required. Soil fumigation, and generally early planting and destruction of weed hosts can assist in controlling this nematode (Jabbari & Niknam, 2008).

*Heterodera cruciferae* causes stunting, chlorosis and reddish colors on leaves (Thorne, 1961). Although crop yields are rarely affected, plant growth can be retarded. An inoculum level of 20 cysts per 100 ml soil is sufficient to cause a strong leaf wilting (McCann, 1981). Initial symptoms of *H. cruciferae* include small and undernourished appearance of the host. During the infection, leaves of the infected plants may wilt or curl, especially during hot weather. In the soil, invaded roots branch profusely, while the taproot remains small. Some plants may die prematurely; the survivors produce loose small heads and discolored root systems. Invasion of infected roots by fungi is a common secondary damage. Circular patches of affected plants may appear in the field. The best diagnostic symptom, however, is the appearance of the pearly white, tan or reddish bodies of females dotting the root surface. Almost half of the cabbage growing areas in Samsun, a middle Black Sea region famous for cabbage production in Turkey, are infested with this nematode. Red and white head cabbage varieties were common and susceptible, but kale was resistant (Mennan & Handoo, 2006).

Although there are some reports on the histopathology and other aspects of host responses to some other members of the Heteroderidae (Mundo & Baldwin, 1983; Cohn et al., 1984; Baldwin & Bell, 1985), histopathological studies of *H. cruciferae* has not been reported. Therefore, the objective of this study was to determine the anatomical changes induced by *H. cruciferae* on the susceptible white head cabbage variety *Brassica oleracea* var. *capitata* subvar. *alba* cv Yalova 1 in Samsun Province, Turkey.

## Materials and Methods

### Soil sampling and nematode extraction

Survey was carried out in Samsun during *Brassica* growing season. Soil and root samples were collected at a depth of approximately 20 cm, from the root zone of infected cabbage crops by using the knowledge of our previous study related to infestation level of cabbage cyst nematode (Mennan & Handoo, 2006). Soil samples were obtained by using a spade and approximately twelve litres of soil were obtained by compositing samples from 5-10 locations within an area of 1 ha of each field, according to Young (1990). All subsamples were mixed thoroughly, and divided appropriately to obtain a representative sample of 1000 ml of soil and roots mix. The soil was immediately placed in plastic bags and transported to the laboratory. Cysts were extracted from air-dried soil and infested root samples by a sieving method (nested 30-60), using the remainder of each composite sample that had been placed in an open paper bag and allowed to air dry thoroughly for about 2 weeks, and then dry soil was used to extract the cysts. A sieving method was used to obtain the cysts, which were counted in 6 cm diameter plastic Petri dishes after collection (Southey, 1986; Golden, 1990).

### Mass culture of *Heterodera cruciferae*

Sandy loam soils (80% sand, 10% silt, 10% clay) used for pot experiments were autoclaved at 121°C for 20 min. Surface-sterilized (with 5% NaOCl) cabbage seeds (*Brassica oleracea* var. *capitata* subvar. *alba*) were mass-germinated, selected for uniformity and transplanted 20 days after germination into pots (500 ml) and watered as needed. About 3-weeks-old cabbage seedlings were transplanted to these pots. The pots were randomly placed in growing chambers set at 25 ± 2°C, 8 h dark and 16 h light, and watered as needed. Cysts obtained from survey were used for mass culture by putting 3 of them on one seedling in 500 ml soil. Waited a sufficient time to have for next generation production, about 8 weeks, cysts were re-extracted as explained above. Cysts were collected with an aspirator under stereobinocular microscope (Leica S6D) (Southey, 1986).

### Experimental design

Cysts were used to obtain sterile eggs. Ten cysts were put into hatching Petri dishes containing kale root exudates (Castillo & Vovlas, 2002) in 16 °C incubator. Hatching dishes were checked every day under stereobinocular microscope and when there were enough newly hatched sterile juveniles, experiments were started as designed.

Sterile susceptible *Brassica* seedlings with 2-3 dicotyledonous leaves in 300 ml of soil in pots were infested with 1,000 juveniles of cabbage cyst nematode in 10 ml sterile water. Yalova F1 variety was used for experiments because it is known that this variety is very susceptible to *H. cruciferae* (Aydınlı & Mennan, 2009) and is commonly preferred for production by growers. Control pots received 10 ml of juvenile free, sterile water. Pots were kept in growth chambers set at 20 ± 2°C, 8 h dark and 16 h light and watered as needed and checked regularly. Experiments were completed with 4 replicates and repeated once.

After 3, 6, 12, 24, 48 and 72 h and 30 days from artificial inoculations, 2 plants were cut off from soil from infected and juvenile free pots and put into FAA fixatives for 10 days. By using sharp needle, small root pieces were obtained and then put into Alcohol-Xylol series for microwave techniques (Askin et al., 1995). Plant tissues were put into different series with different times (Table 1). Samples were embedded with liquid paraffin at about 50 °C. Thick serial sections (10-15 µm) were then cut on a rotary microtome and stained with 1% acid fuchsin, and examined under a light microscope and photomicroscoped (Nikon Eclipse 600).

Table 1. The series of solutions used in Microwave Protocols for Paraffin Microtechnique to stain and fix *Heterodera cruciferae* within the roots of *Brassica oleracea* var. *capitata* subvar. *alba*.

Ingredients	Duration (Min)
Xylol	5
Xylol	5
40% Alcohol	5
96% Alcohol	5
70% Alcohol	3
Acid fuchsin (1%)	3
70% Alcohol	2
96% Alcohol	2
Isopropyl Alcohol	2
Xylol	2

## Results and Discussion

Several morphological changes were observed in root tissues of infected *Brassica* plants even 3 hours after inoculations (Figures 1 and 2).

Second stage juveniles directly penetrated through vascular system became sedentary and started to feed. Nematode penetration and migration within the roots was intracellular. Some larvae were positioned entirely within endodermal cell layers, while tails of other larvae were partially extended three or four cell tiers into the cortex or were exposed on the root surface. The head regions of feeding nematodes were within or close to the endodermal cells and were oriented toward the vascular system (Figures 2A-H).

After reaching their feeding positions, most larvae became oriented parallel to the root axis, but some were in diagonal positions within the cortex (Figures 1E, F, H). Most larvae had completely penetrated the root tissue and frequently traversed a root tip adjacent to the root cap. Broken cells were often necrotic (Figure 1D). The cytoplasm was granulose, slightly dense, and with a large vacuole in some cells, and nuclei were spherical, somewhat hypertrophied, with prominent nucleoli. After 24 hours of feeding, multinuclear feeding site was became “syncytia” (Figure 2).

Cell walls generally thickened and interrupted in some sectors, thus cytoplasm moved through neighboring cells. The syncytia cytoplasm was dense, of granular aspect, with different degrees of vacuolization and with large-sized nuclei of spherical, ovoid, or lobulated shape and prominent nucleoli. Walls adjacent to xylem vessels usually showed development of irregular wall thickenings and of rugose texture. Dead cells showed different levels of disorganization and were non-functional especially 30 days after inoculations (Figure 2). White females were also observed around the roots, 30 days after inoculations (Figure 2F-G).

Thirty days after inoculations, above and below ground symptoms were also obtained (Figures 3A-B). Retardation of growth was observed in the infected treatments. The heights of shoot of infected plants were less than healthy untreated control. Root proliferation was also obvious in infected plants. Among above ground symptoms, plant height and total leaves were more obvious. Plant height in infected plants was much lower than untreated control plants, and uninfected plants had moderately more leaves than infected plants. Root proliferation was conspicuous in infected plants; although root colour was the same, roots were shorter in infected plants.

*Heterodera cruciferae* was first determined on the roots of *Brassica* sp. in 1963 in eastern part of Turkey (Erzurum) (Yüksel, 1966a; Yüksel, 1966b; Yüksel, 1973). It is widespread in Turkey and almost all over the world where *Brassica* is grown. Above ground symptoms of damage by cyst nematodes in *Brassica* are usually shown as patches of pale and stunted plants. The symptoms of the aerial parts of plants infected with these nematodes can be identical with the symptoms of plants sustaining severe nitrogen and other mineral deficiencies. Plants attacked by the nematodes wilt readily in dry weather, as a consequence of damage to the root system. Although, there is no information available related to anatomical changes caused by cabbage cyst nematode even on other hosts, there is some research about the histopathological changes of *H. glycines* on different hosts (Endo, 1992) soybean (Acedo et al., 1984; Young et al., 1999; Marcelo et al., 2008), *H. mediterranae* on olive roots (Vovlas & Inserra, 1983) and *H. schachtii* on sugarbeet (Yu & Steele, 1981; Inserra et al., 1984). In general, sedentary nematodes are destructive plant pathogens and can cause significant yield losses. In the roots of their host plants, cyst nematodes and root-knot nematodes induce different, highly specialized feeding sites—syncytia and giant cells, respectively—to optimize nutrient uptake (Hoth et al., 2008).

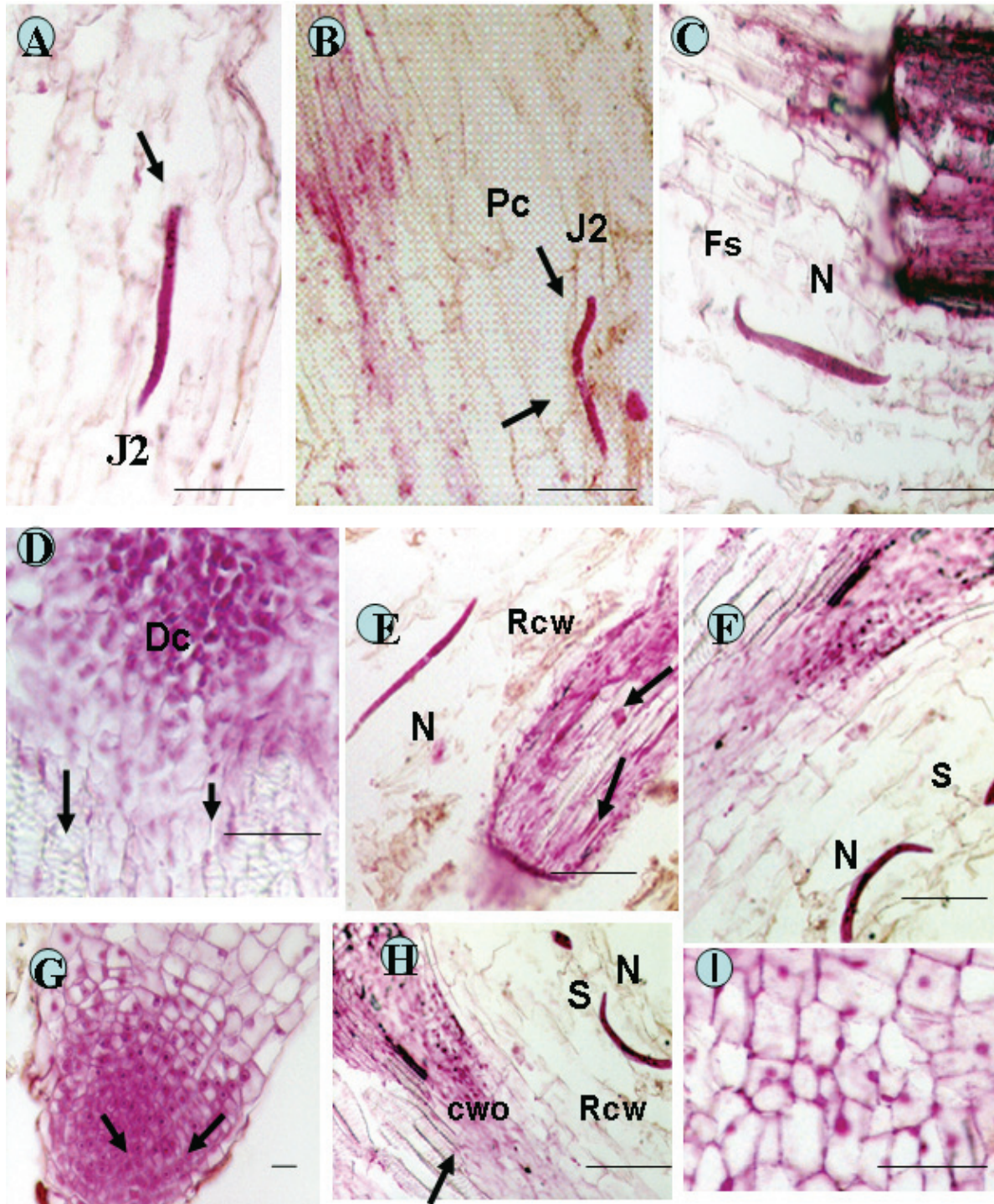


Figure 1 A.- C). Second stage juvenile penetrating into root tissue, head of nematode was inside, tail was outside, Scale bar = 100  $\mu$ m. D-H) Second stage juvenile inside the stele feeding on the cell, Note dark tissue elements in neighbor cells, arrows indicating the pathways of juveniles, and feeding on a syncytium originating from an endodermal cell causing openings cell wall and ruptured wall around the feeding site, Note necrosis along path of nematode penetration (arrows), I) Juvenile free cabbage root tissue. Scale bar = 100  $\mu$ m, J2= Second stage juvenile, Pc= Partioned cells, Fs= Feeding site, N= Nematode, Dc= Dense cytoplasm, Rcw= Ruptured cell wall, S= Syncytium.

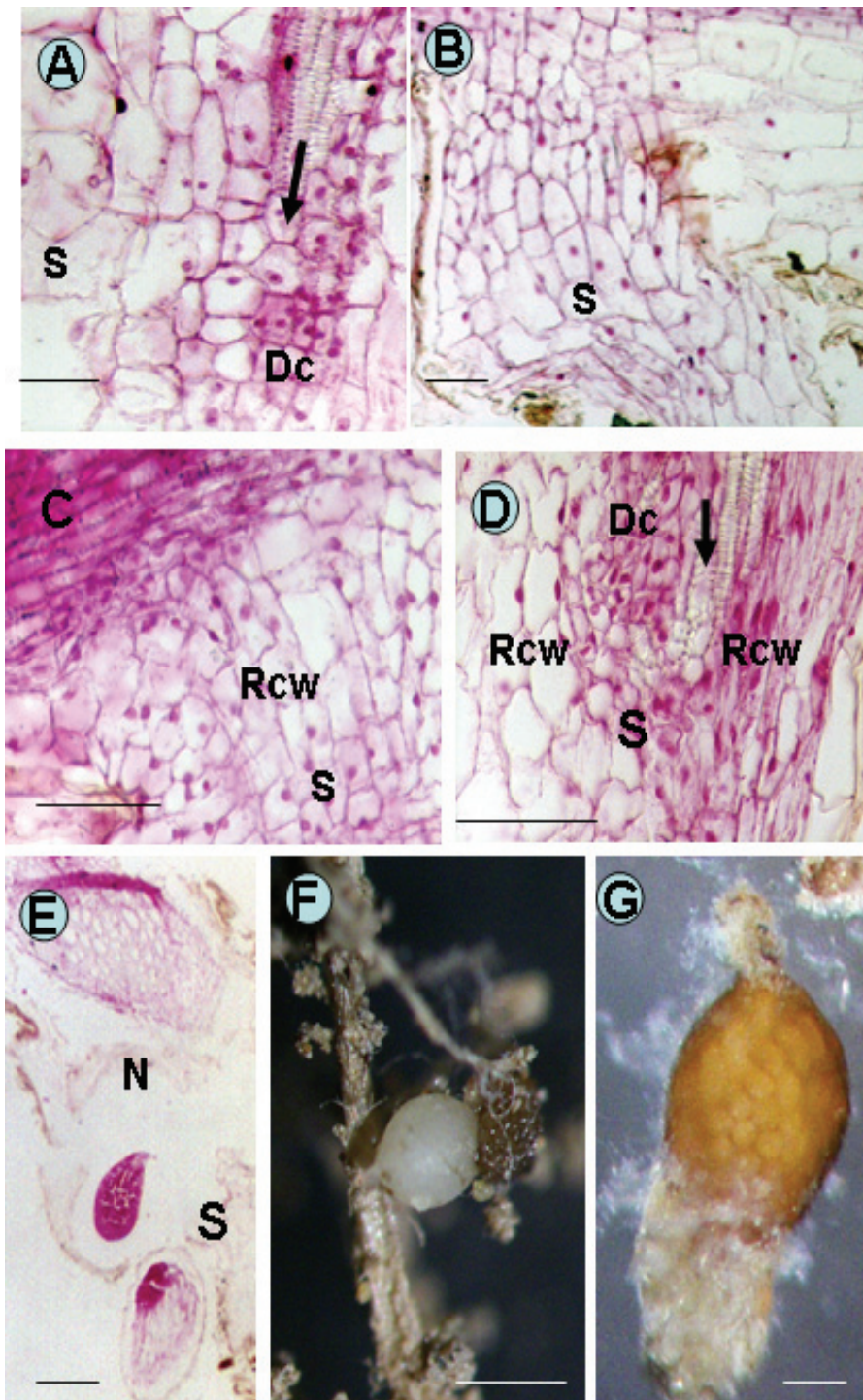


Figure 2. Anatomical alterations caused by *Heterodera cruciferae* in *Brassica oleracea* var. *capitata* subvar. *alba* roots. A) Arrows indicating opening resulting in entrance of second stage juveniles, scale bar = 25  $\mu$ m. B) Second-stage infective juvenile embedded in the cortex and feeding on a syncytium, 24 h after inoculation, scale bar = 50  $\mu$ m. C) Infested cells with dense cytoplasm dark color and ruptured cell walls 24 h after inoculations, scale bar = 50  $\mu$ m. D) Ruptured cell walls and dense cytoplasm around syncytium, scale bar = 50  $\mu$ m. E) Dark colored cells associated with nematode feeding compared with light, non-damaged cells and young female nematode around syncytium, scale bar = 200  $\mu$ m. F) White female under light microscope, head inside the root tissue, remainder of body protruding from cells, 30 days after inoculation, scale bar = 250  $\mu$ m. G) Young cyst of *Heterodera cruciferae* containing egg mass, scale bar = 100  $\mu$ m. S= syncytium, Dc= Dense cytoplasm, Rcw= Ruptured cell wall, N= Nematode.

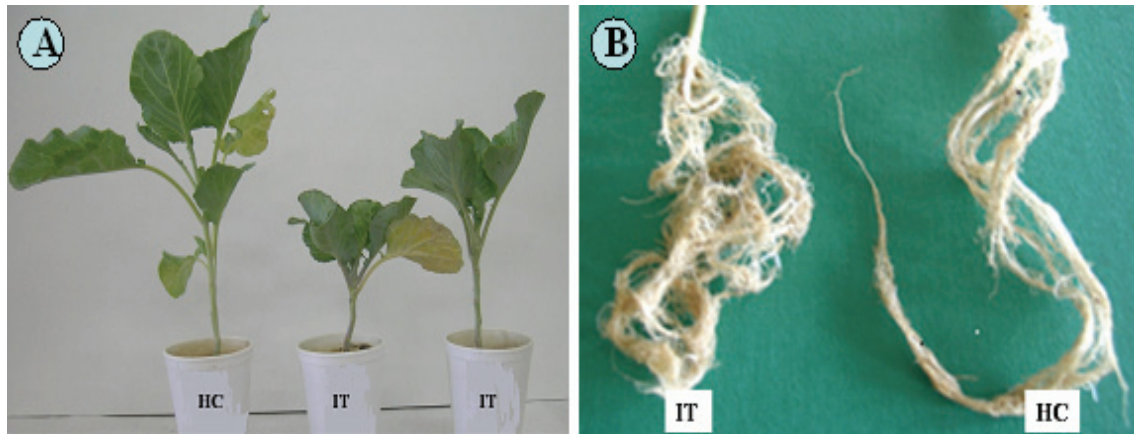


Figure 3. A) Above- and B) below-ground morphological changes in *Brassica oleracea* var. *capitata* subvar. *alba* infected with 1,000 juveniles of *Heterodera cruciferae*. HC= Healthy Control, IT= Infected treatment.

It is known that after *Heterodera* infection, the vascular system is altered. (Magnusson & Golinowski, 1991; Wyss & Grundler, 1992; Golinowski et al., 1996). After penetration, second stage juveniles directly go through vascular system, remain sedentary and start feeding. After 24 hours of feeding, multinuclear feeding sites are called “syncytia”. Meanwhile, neighboring cell walls perforate and contents of cells collect (Wyss & Grundler, 1992). According to Bird (1974), the second stage larva of *H. cruciferae* takes about 5 minutes to cut a slit in a plant cell wall large enough for it to pass through. Thus, the process of entry into a plant can be quite rapid. Our results were in accordance with that finding and we found lots of changes in root tissues, even at our first examination time.

It is known that infested cell walls are slightly thickened and show interruptions in some sectors, allowing cytoplasm movement between neighboring cells. The cytoplasm was granulose, slightly dense, alveolar and contained small globules with a large vacuole in some cells, and nuclei were spherical, somewhat hypertrophied, with prominent nucleoli (Vovlas & Inserra, 1983; Tordable et al., 2003; Doucet et al., 2008).

They established permanent feeding sites in the endodermis by modifying endodermal and adjacent pericycle cells to form a syncytium. Pericycle cells enlarged, and the cytoplasm became very dense. Phloem and vascular parenchyma cells were also incorporated into the syncytium. Metaxylem elements sometimes fused with syncytium but more often were compressed by the syncytium expansion. During cell fusions, walls of adjacent cells dissolved, leaving only wall segments inside the syncytium. Syncytial nuclei were usually hypertrophied, deeply indented, and with prominent nucleoli. There was no evidence of mitotic activity inside the syncytia (Jones, 1981; Suarez et al., 1985).

The syncytium was typically formed within the stele and was limited on the side toward the nematode by endodermis or in part by cortical cells. The reticulate hyperchromatic cytoplasm was continuous throughout the syncytium and contained enlarged nuclei with distinct large nucleoli. These findings are in agreement with results of other investigators (Mankau & Linford, 1960) who reported that the syncytium was formed by a progressive dissolution of adjoining cell walls.

We observed white mature female 30 days after inoculation in *Brassica* roots. This study revealed anatomical changes induced by the cabbage cyst nematode, *Heterodera cruciferae* on susceptible *Brassica* roots for the first time.

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