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Effect of Acetylsalicylic Acid on the Reproduction of Soybean Cyst Nematode in Susceptible Soybean

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감수성 콩에서 Acetylsalicylic Acid의 콩씨스트 선충 증식 억제 효과

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ABSTRACT: Reproduction of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe on the susceptible soybean cultivar, Lee 74, was significantly reduced by pre-, post- and simultaneous treatments of acetylsalicylic acid (ASA, aspirin). The control efficiencies were 60%, 64% and 87% for pre-, post- and simultaneous treatments, respectively. ASA had no significant effect on the survival of 2nd stage juveniles and their penetration into the soybean root tissues, but significantly inhibited the early stage nematode growth in the roots. Syncytia were formed 2~3 days after inoculation in the susceptible soybean without ASA treatment, characterized by dense cytoplasm and increased cellular organelles such as mitochondria and endoplasmic reticulum. The nematode stylet was penetrated into the syncytial cell, and feeding tube was formed at the nematode stylet entry. However, in the ASA treatments, syncytium was not formed or degenerated, depending on the root tissues. In the pre-treatment of ASA, nematode stylets did not penetrate into cells, showing callose-like cell wall thickening formed at the nematode probing sites, or retracted from the infected cells. The stylet penetration sites of syncytial cells appeared to be sealed off with fibrillar materials. With post-treatment of ASA, syncytia formed by the nematode were degenerated, characterized by degradation of syncytial cytoplasm.

***Key words*: acetylsalicylic acid, soybean cyst nematode, reproduction, ultrastructure.**

One of the various effects of salicylic acid (SA) and its derivatives such as acetylsalicylic acid (ASA, aspirin) on plants is the induction of resistance to plant pathogens (18). SA induces resistance of hosts to viral (1, 23, 24), fungal (6, 17, 25), and bacterial (19) infections. However, no study has been reported about the SA-induced resistance to the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe in soybean, *Glycine max* (L.) Merr.

Hypersensitive reaction (HR) is a common form of disease resistance in plants, and SA is known to affect several processes associated with HR such as fortification of plant structural defenses by synthesis and incorporation of lignin, hydroxyproline-rich glycoproteins and phenolic materials into cell walls (16). Increase of peroxidase activity is also related to HR, which is necessary for lignin biosynthesis, cross-linking of cell wall proteins and suberization (13, 14, 16). The holistic mechanism of resistance to plant parasitic nematodes suggested by Giebel (7, 8) is similar to HR in some biochemical processes. In SCN, callose-like cell wall thickening was suggested as HR response of soybean (4). Cell wall thickening with or without necrosis has been related to resistance mechanism, inhibiting the development of a syncytium that serves as nutritional sources for the infecting nematode (12, 21).

In this study, induction of resistance in soybean to SCN by ASA was studied by investigating the effects of ASA on the nematode survival, penetration, growth and reproduction, and subcellular changes of the infected root tissues.

MATERIALS AND METHODS

Plant and nematode inoculation. A soybean cultivar susceptible to all known SCN races, Lee 74, were used in this experiment. Soybean seeds were planted in vermiculite, and seedlings were transplanted into 7.5-cm-di-

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ameter clay pots with sterilized river sand 5 days after sowing. Second-stage juveniles (J2) of SCN race 14 collected from cysts by Baermann funnel method were used for the nematode inoculum. Inoculation of the nematode followed basically the previous studies (9, 11), by pouring SCN J2 suspension into the soybean rhizosphere in each pot. About 300 J2 were applied for a pot throughout the experiment, unless otherwise mentioned.

Effect of ASA on survival and reproduction of SCN. As 2.5 mM or 5.0 mM of ASA was effective in the induction of resistance in cucumber (6), a similar range of ASA concentrations was used in our experiment. ASA was dissolved in water to make 4 mM solution, and the ASA solution was diluted serially to 2 mM, 1 mM and 0.5 mM. SCN J2 (approximately 1,000 nematodes) were placed in each diluted ASA solution for 2 hours, and then placed on a funnel with ASA solution of the same concentration for each treatment. After 24 hours, SCN J2 recovered by the Baermann funnel method (only living nematodes can move down to the bottom of the funnel) were collected from the funnels, and the number of active nematodes was counted under a stereomicroscope (x 60). Each treatment had 3 replicates.

For each SA concentration, about 50 ml solution was poured into the soil with a soybean plant in a 7.5-cm-diameter clay pot 2 days after inoculation. Thirty days after inoculation, cysts on roots and in soil were collected by rubbing and sieving through nested 20-mesh and 60-mesh sieves, and the cysts were counted under the stereomicroscope (x 30). Four replicates were used for each treatment.

Effect of ASA on SCN penetration and development. As 2.0 mM ASA was the optimum concentration for the study of ASA effect on SCN in this experiment, 2.0 mM of ASA was used for further experiments. About 50 ml ASA solution was treated 2 days before, 2 days after and at the same time of nematode inoculation. Influence of ASA on nematode penetration and growth at the early stage of infection was examined by staining the nematodes in soybean roots. Staining of the nematodes in roots followed one of the staining methods (22) using bromophenol blue as described in other papers (10, 11). The stained roots were rinsed with 50 % ethanol, and the number of the juveniles was counted under the stereomicroscope (x 60). The invading juveniles were divided into two groups; second stage juveniles (J2) without swelling, and swollen juveniles by growth. Thirty days after inoculation, cysts formed in each pot were collected by sieving through 20-mesh and 60-mesh sieves, and counted under the stereomicroscope. Four replicates were used for each treatment.

Electron microscopy. Soybean root segments infected with SCN were processed for electron microscopy. Soybean roots were dissected about 2 mm long, and fixed in Karnovsky's fixative for 4 hours, post-fixed in 1% osmium tetroxide for two hours, dehydrated in a ethanol series, and embedded in Spurr's epoxy resin (23). Embedded materials were sectioned 80~90 nm in thickness with a diamond knife. The sections were stained with uranyl acetate and lead citrate, and observed under a JEOL 100 CX electron microscope. For each treatment, more than 4 specimens were examined with the electron microscope.

RESULTS

Effect of ASA on SCN survival and reproduction. There was no difference in the survival of SCN J2 between the control and the ASA treatments (up to the 4.0 mM concentration), while nematode reproduction was decreased significantly with 2.0 and 4.0 mM ASA treatments (Table 1). Soybean plants were withered at 4 mM, but recovered later. No noticeable wilt symptom was induced on the aboveground parts of the soybean plant by ASA at no more than 2.0 mM ASA concentrations. However, growth was somewhat retarded at 2 mM.

Effect of ASA on SCN penetration and development. SCN penetration was not significantly affected by 2

Table 1. Effect of acetylsalicylic acid (ASA) on the survival and reproduction of soybean cyst nematode

Concentration of ASA (mM)	No. nematodes (J2) recovered/funnel[a]	No. cysts/pot[b]
0.0	768 X[c]	57.3 (100) X
0.5	964 X	52.0 (91) X
1.0	730 X	45.3 (79) X
2.0	790 X	14.0 (24) Y
4.0	805 X	1.0 (2) Z

[a] About 1,000 second stage juveniles (J2) were placed in each ASA solution for 2 hours, and placed onto the funnels. Twenty four hours later, J2 were recovered from the funnels. Numbers are averages of three replicates.

[b] Fifty ml of each ASA solution was poured onto soil with a plant in a 7.5-cm-diameter pot 2 days after inoculaiton of about 300 SCN J2 per pot. Thirty days after inoculaiton, cysts were collected through 20-mesh and 60-mesh sieves. Numbers are averages of four replicates. Numbers in parentheses are percentages of the number of cysts relative to that of control (0.0 mM).

[c] Means within a column followed by the same letter do not differ significantly according to the Duncan's multiple range test.

Table 2. Effect of acetylsalicylic acid (ASA) on the penetration, growth at the early stage of infection, and reproduction of soybean cyst nematode with different time of application[a]

Treatment ASA (mM)	Penetration[b] (No. J/root)	Growth[c] (% swollen J/root)	Reproduction (No. cysts/pot[d])
Control	76.0 X[e]	60.3 X	38.5 (100) X
Pre-	68.7 X	34.9 Y	15.5 (40) Y
Post-	62.7 X	25.5 YZ	13.8 (35) Y
Simultaneous	60.0 X	14.4 Z	5.0 (12) Y

[a] About 300 second stage juveniles (J2) were inoculated on the rhizosphere of soybean plant 2 days before (post-treatment), 2 days after (pre-treatment), and at the same time (simultaneous treatment) of 0.2 mM ASA.

[b] Nine days after inoculation, soybean roots were stained with 1% bromophenol blue, and the number of juveniles (J) was examined. Numbers are averages of 3 replicates.

[c] Sausage-shaped and obese juveniles (J) were counted.

[d] Cysts formed 30 days after inoculation. Numbers are averages of 4 replications. Numbers in parentheses are percentages of the number of cysts relative to that of control.

[e] Means within a column followed by the same letter do not differ significantly according to the Duncan's multiple range test.

mM ASA, but SCN development was significantly retarded and the reproduction was reduced by ASA treatment (Table 2). There was somewhat differences in the percentages of the swollen nematodes among pre-, post- and simultaneous treatments; however, reproductions were not significantly different among the treatments. The control efficiencies for pre-, post- and simultaneous treatments, were 60%, 64%, and 87%, respectively.

Electron microscopy. Syncytia were always formed in soybean root tissues in no ASA treatment at 2~3 days after inoculation. The cytoplasmic features of syncytial cells induced by the nematode with no ASA treatment were characterized by dense cytoplasm, small vacuoles and dissolved cell wall (Fig. 1). Nematode stylet was penetrated into the syncytial cell, and a feeding tube was formed adjacent to the stylet (Fig. 2).

In all specimens with ASA treatments, stylets were placed outside of cells, showing no penetration into the cells or retraction of the stylets from the cells adjacent to nematode invasion (Figs. 3~8). In pre-treatment of ASA, cytological features varied depending on soybean root tissues examined. Without stylet penetration, syncytium was not formed, and no structural modification occurred except secondary wall thickening formed probably by nematode probe (Fig. 3). Structural modifications were noticed in the invaded cell characterized by breakdown of vacuole and increased cytoplasm (Fig. 4). Sometimes large syncytial cells with hypertrophied nucleus and increased cellular organelles such as endoplasmic reticulum (ER), mitochondria and plastids were formed by the nematode infection (Fig. 5). However, in this case the stylet was also retracted from the infected cell. Around feeding plug fibrillar materials appeared to be accumulated, and vesicles sometimes containing electron-dense materials, were prominent and appeared to be associated with deposition of the materials (Figs. 6, 7).

In post-treatment of 2 mM ASA, syncytia were formed, but all of the syncytia examined were necrotized and degenerated, which was characterized by dark and degraded syncytial cytoplasm (Fig. 8). Plasmalemma was detached from the cell wall.

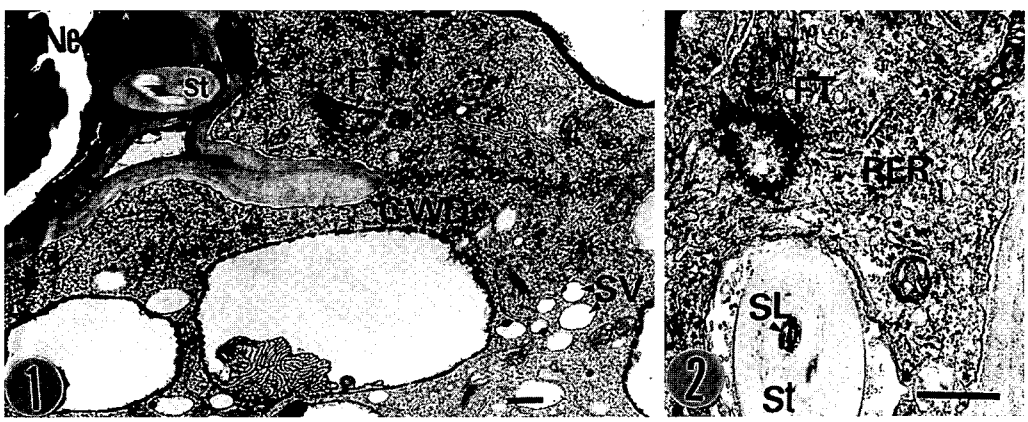

Fig. 1. Syncytium formed 56 hours after inoculation in the root tissue of a susceptible soybean cultivar, Lee 74, without acetylsalicylic acid (ASA) treatment, showing dense cytoplasm, cell wall dissolution (CWD), and increased small vacuoles (SV). FT=feeding tube, St=stylet, Ne=nematode. Bar=1 μm.

Fig. 2. Higher magnification of the nematode stylet (St) penetration site in Fig. 1, showing details of stylet penetration and dense cytoplasm. FT=feeding tube, RER=rough endoplasmic reticulum, SL=stylet lumen. Bar=1 μm.

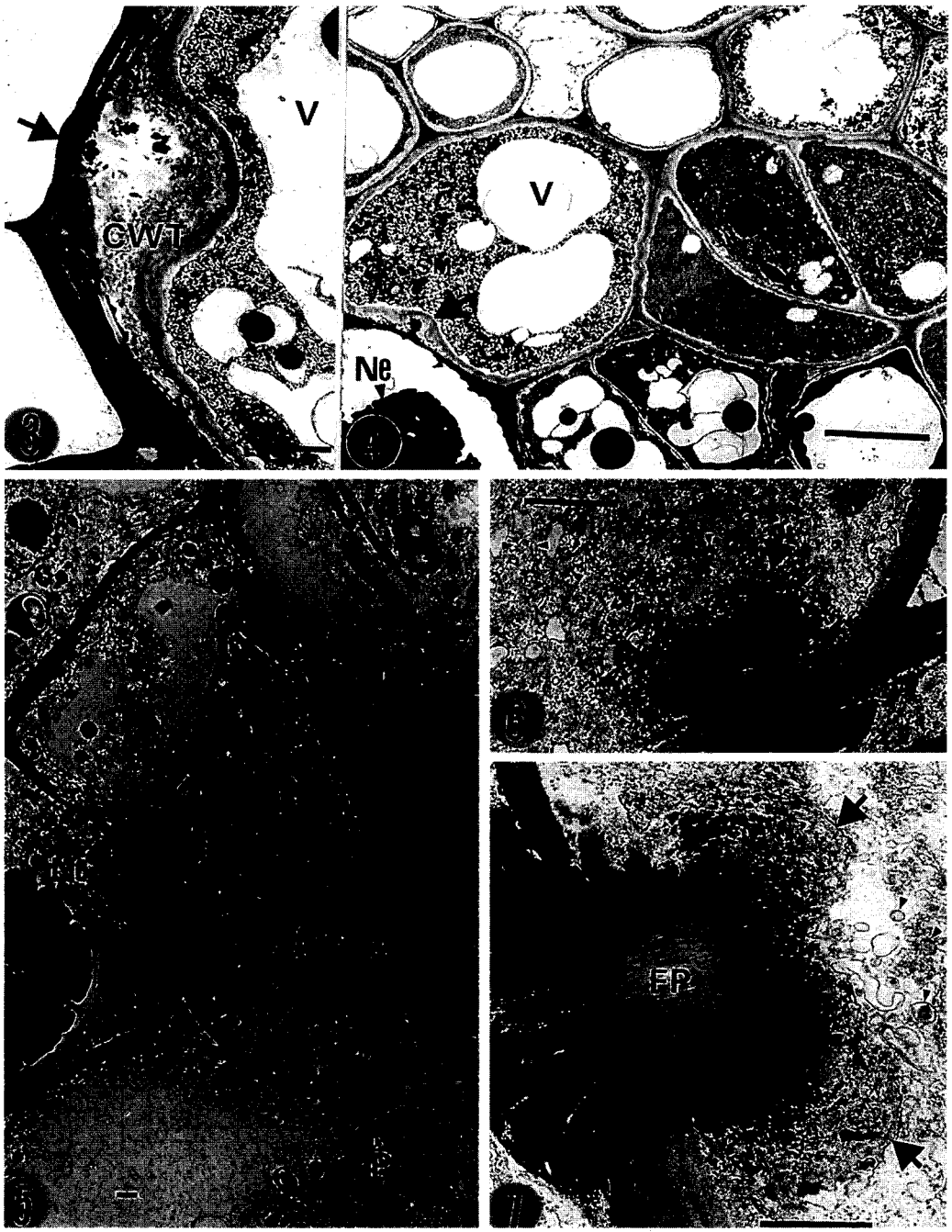

Fig. 3. Callose-like cell wall thickening (CWT) formed in the middle lamella at the nematode penetration sites (arrow) 56 days after inoculation in Lee 74 infected with soybean cyst nematode and pre-treated with 2.0 mM ASA. Note there is no indication of stylet penetration into the cell. V=vacuole. Bar=1 μm.

Fig. 4. Thickening of cell wall (arrow) at the stylet penetration site 56 days after inoculation in Lee 74 pre-treated with 2.0 mM ASA. Nematode secretion materials (SM) indicates that the nematode stylet have penetrated into the cell. Note the hypertrophied cell with breakdown of vacuole (V). Ne=nematode. Bar=10 μm.

Fig. 5. Structure features of syncytial cells (characterized by cell wall breakdown and hypertrophied cells) formed by the soybean cyst nematode infection in soybean root with pre-treatment of 2.0 mM ASA (56 hours after inoculation). Note accumulation of materials (arrow) around nematode feeding plug (FP). M=mitochondria, Ne=nematode, N=nucleus, P=plastid, SM=nematode secretion material. Bar=1 μm.

Figs. 6 & 7. Higher magnifications of the nematode penetration sites, showing details of accumulation of fibrillar materials and feeding plug (FP). Note abundant vesicles (arrowheads) around the feeding plug in Fig. 6. Bar=1 μm.

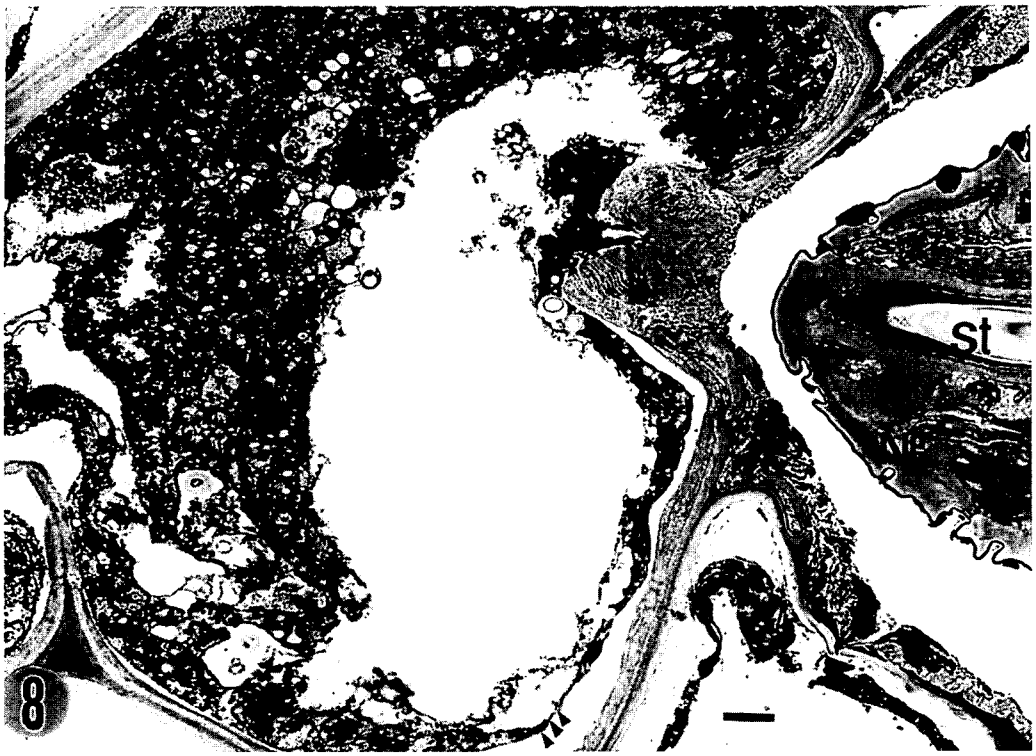

Fig. 8. Degeneration of syncytial cells formed by the nematode infection 56 hours after inoculation in Lee 74 with post-treatment of 2 mM ASA. Syncytial cytoplasm is degenerated and necrotic. Plasmalemma is detached from the cell wall (arrowheads). Ne=nematode. St=stylet. Bar=1 μm.

DISCUSSION

Salicylic acid (SA) belongs to a group of plant phenolics. Acetylsalicylic acid (ASA, aspirin), one of its derivatives, is hydrolyzed to SA in aqueous solutions, and exogenously applied ASA is rapidly converted to SA in animal and plant tissues (18). SA and its derivatives reduced the gall formation and reproduction of a root-knot nematode species (15). In our study, ASA treatment inhibited SCN growth and reproduction; however, it influenced neither on the survival of SCN nor on the nematode penetration into soybean root tissues, suggesting that the toxic effect of the chemical on the nematode *per se* may be minimal. Thus, some biochemical changes of the plant by ASA treatment and/or ASA (or SA) itself might hinder nematode feeding, which inhibited the nematode growth in the soybean root system.

In ASA treatment, a large portion of the soybean root system appeared to be degenerated, and soon after replaced by new roots which appeared more stout than the non-treated roots. In the post-treatment of ASA, cytoplasmic degeneration occurred in the syncytial cells upon which the nematode depends for growth. Cellular degeneration also occurred in the uninfected cells, suggesting that such cellular degeneration may not specifically confined to the syncytial cells.

In the pre-treatment, the ultrastructure aspects of the infected tissue varied depending on the stylet invasion. The formation of callose-like secondary wall thickening was observed adjacent to the nematode with no stylet invasion into the cell. The wall thickening may reflect the enhanced defense activity against the nematode because it probably served as a mechanical barrier against the stylet penetration.

SA is known to affect several processes related to hypersensitive reaction, one of which is the synthesis and incorporation of lignin, hydroproline-rich glycoproteins and phenolic materials into cell walls, fortifying the plant's structural defenses (16). SA also increases peroxidase activity which is necessary for lignin biosynthesis, cross-linking of cell wall proteins and suberization (19). Accumulation of some fibrillar materials around feeding plugs by ASA treatment in our experiment may be an indication of the wall material synthesis.

In the cells with structural modification or in syncytial cells, stylet vestiges were found, indicating that

the nematode stylets had penetrated into the cells. However, the nematode stylets were always retracted from the infected cells (or the syncytial cells) in ASA treatment. SA is one of phenolic compounds which have been associated with chemical defenses of plants against microbes, insects and herbivores, and nematodes might evade feeding because of ASA (SA) and/or such chemicals or H_2O_2 induced by the ASA treatment (2).

SA and ASA induce the production of pathogenesis-related (PR) protein and resistance to TMV in tobacco (1, 23, 24). One of PR proteins, group 5a, has similar degree of homology with a maize protein that is a bifunctional inhibitor of α-amylase and proteases of insects (20), and is related to plant defense against insects. Thus, the PR 5a protein may be one of the materials that are related to the SCN inhibition.

In soybean cultivars resistant to SCN, syncytia are formed by the nematode infection, but degenerate early. Structural modifications related to the syncytial degeneration were suggested to be the formation of cell wall apposition, necrotic layer, and nuclear degeneration (12, 21). In ASA treatment, such cellular modifications as in soybean cultivars resistant to SCN were not found in this experiment. However, accumulation of secondary materials around the feeding plug probably indicates sealing-off of the feeding plug. Feeding plug is needed for sustaining a stylet at the penetration site and stylet reinsertion to resume feeding after molting of the nematode (3, 5). Therefore, this structural modification may be related to the block of the nematode feeding.

요 약

Acetylsalicylic acid(ASA)를 선충 접종전후 또는 접종과 동시에 감수성 콩 품종 Lee 74에 처리한 결과 콩씨스트 선충의 증식이 크게 감소하였다. 접종전후 또는 동시 처리에 의한 선충의 방제가는 각각 60%, 64% 및 87%이었다. ASA는 2기 유충의 생존과 콩 뿌리 침투 억제에는 유의적인 효과가 없었으나 뿌리 내에서 선충의 성장을 억제하였다. ASA를 처리하지 않은 콩에서는 접종 2~3일 후에 세포질이 농후해지며 미토콘드리아와 소포체 등 세포 소기관이 증가하는 특징을 보이는 병합체(syncytium)가 형성되었고, 선충의 구침이 이들 세포를 침입하고 흡수관(feeding tube)이 구침 침입 부위에 형성되어 있었다. 그러나 접종전 ASA를 처리한 콩에서는 선충의 구침이 세포를 침입하지 않았거나 침입 부위에서 빠져 나와 있었다. 구침의 침입이 없었던 곳은 구침 주변 세포의 세포막이 비후해져 있었고 구침이 침입했던 부위는 섬유상의 물질이 축적되어 막혀지는 것으로 보였다. 접종후 ASA를 처리할 경우에는 병합세포(syncytial cell)의 세포질이 죽어 병합체가 퇴화하였다.

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