JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 485-492

Effects of Different Jasmonic Acid Mutant Rootstocks on Root-knot Nematode and Soil Microbiology in Grafted Tomato

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(Received: 12 April 2014; accepted: 09 May 2014)

To explore the effect of grafting and jasmonic acid on root knot nematode disease, and determine the function of jasmonic acid during grafting on root knot nematode resistance, JA biosynthetic mutant (*spr2* plants) and JA-overexpression transgenic plants (*35S::PS* plants) of tomato were used as rootstocks, wild type of autologous grafting plants as a control, the resistance to root knot nematode between various varieties and the result of grafting on soil microorganism were analysed. The results showed that a total microorganism quantity in *spr2* was significantly less than that in *35S::PS* plants. Grafting on different rootstocks combinations of WT/ *spr2*, WT/*35S* made soil microbial aggregates more than WT/WT. Furthermore, the amount of fungi, actinomyces, and bacteria in grafted plants of WT/*35S::PS*, was more than WT/WT with a significant difference. Above results suggested that the grafting and jasmonic acid synthesis changed the rhizospheric microorganism amount and composition of tomato, increased the amount of beneficial microorganism, thereby improving the resistance to root knot nematode.

Key words: Tomato; Jasmonic acid mutants; Soil microorganism; Root knot nematode.

Tomato (*Lycopersicon esculentum* Mill) is one of the greenhouse vegetables which are widely cultivated in the word. In recent years, the phenomenon of continuous cropping obstacle in tomato cultivation has been added because of the soil structure adjustment, decreased cultivation area and the increased multiple cropping index (MCI). Continuous cropping converts soils from being bacteria-dominated to fungi-dominated. Fungal dominance is an indicator of soil fertility exhaustion (Acosta-Martínez *et al*., 2010; Guo *et al*., 2011). Continuous cropping is also the major cause of severe damage from root-knot nematode, which is estimated to cause approximately $100 billion of annual crop loss worldwide (Zhang *et al*., 2008; Overstreeta *et al*., 2010). Tomato is sensitive to root-knot nematodes. Once infected, the tomato plant is difficult to cure with pesticides (Terefe *et al*., 2009; Fujimoto *et al*., 2011). Jasmonic acid (JA) is a well-recognized signal that plants release against pests. When plants are subjected to external aggressors, JA can reach a high level in a short time to induce a systemic resistant response (Senthil-Nathan *et al*., 2009). Foliar applications of exogenous JA can influence the expression of protease inhibitors in distant leaves to increase the plant's resistance to pests. JA can also reduce the reproductive capacity of non-lethal nematodes in susceptible tomato plants and increase the effect

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of nematode-resistance genes at high temperature (Feng *et al*., 2007).

With the increasing duration of continuous cropping, the proportion of fungi gradually increases, the proportion of soil actinomycetes drastically increases, and the proportion of soil bacteria significantly decreases (Eekeren *et al*., 2008). After years of continuous potato cropping, the soil microorganisms retain a high capability to utilize single carbon substrates, and the soil microbial community also retains highly versatile functions in carbon utilization. After constant potato cropping, the bacteria-to-fungi ratio of soils decreases by 9.18-32.11% compared with control soils, indicating that continuous cropping can change soils from being bacteria-dominated to fungi-dominated (Ma *et al*., 2010). A plant's resistance to soil-borne diseases is closely related to the number and composition of the rhizospheric microorganisms. Disease-resistant plant varieties have significantly more fungi and actinomycetes in the rhizosphere than do the susceptible varieties. Cai *et al*. found that three types of microorganisms (bacteria, actinomycetes, and fungi) were significantly more abundant in the rhizosphere of diseased pepper plants than in that of healthy plants (Cai *et al*., 2003). Grafting is a commonly used method to overcome the problems of soils in continuous cropping. Grafting can efficiently improve plants' resistance to adversity. Wang *et al*. reported that grafting increased the numbers of rhizospheric actinomycetes and fungi and decreased the number of rhizospheric bacteria for eggplant plants, and a relatively more rhizospheric actinomycetes and high ratio of actinomycetestofungi among grafted eggplant plants was closely associated with improved disease resistance (Wang *et al*., 2005).

Although many studies have used exogenous JA or grafting to increase tomato plants' disease resistance, few have examined the correlation between the grafted plants' resistance and JA.There are even fewer studies on the mechanism by which JA improves tomato plant's resistance to root-knot nematodes when grafting JA mutants to wild-type (WT) tomato plants. Hence we utilized the JA biosynthesis-defective mutant *spr2* and JA-overexpression transgenic tomato plants (*35S::PS*) as rootstocks, WT plants as scions, autologous grafted WT tomato plants as controls to analyse the effect of JA and grafting on the number and species of rhizospheric microbes of grafted tomato, and the role of JA in tomato resistance to root-knot nematode was also explored.

MATERIALS AND METHODS

Experiment materials and design

Tomato cultivar CM (*Lycopersicon esculentum* var Castlemart, WT), *35S::PS*, and *spr2* were used as materials. The original seeds of CM, *spr2*, and *35S::PS* were kindly provided by Professor Chuanyou Li of the Genetics and Developmental Biology Institute of Chinese Academy of Sciences. UC82 were kindly provided by Professor VM Williamson of the University of California, Davis, USA.

The experimental materials were planted in the spring and summer of 2011 in the greenhouse at the experimental field of the Beijing University of Agriculture. *Meloidogyne incognita* was inoculated when the seedlings had four fully extended true leaves. Some of the seedlings were inoculated with *M. incognita* and then sprayed with methyl jasmonate (MeJA). The specific process was first to dissolve MeJA (Sigma-Aldrich Company, 392,707) in ethanol to a concentration of 100 mM and then dilute it to 0.5 mM with water. MeJA solution or carrier solution (5% ethanol) was sprayed on the plant leaves immediately after inoculating the tomato roots with 5,000 second-instar incognita. Plants sprayed with water were used as a control, and the root-knot nematodes were counted 30 days later.

The grafting experiment was designed as follows: WT plants (scion) were grafted to mutant plants (rootstock) and their own roots. Plants grafted to their own roots were used as the controls in the experiment. Cleft grafting was used. Then, root-knot nematodes were inoculated into the surviving grafted plants. Soil samples were collected 6, 24, and 144 h after inoculation for microbiological culture and the investigation of the species and quantity of rhizospheric microorganisms.

Experimental method

Quantification of root-knot nematodes

For root tissue staining and microscopic observation, sodium hypochlorite–acid fuchsin staining was used to identify the species (Bybd *et*

al., 1983). Fine roots were soaked in 1.5% NaOCl for four minutes, washed for 45 s with natural water, and then soaked in distilled water for 15 minutes to remove residual NaOCl. Slightly dry roots were then placed in a beaker with 30 mL of distilled water and 1 mL of acid fuchsin solution (3.5 g of acetic acid fuchsin was dissolved in 250 mL of acetic acid; then, 750 mL of distilled water was added and mixed) was added and boiled for 30 s. The stained roots cooled to room temperature and were taken out and rinsed with tap water to remove the staining agents on the surface. The washed roots were placed in 20-30 mL of acidified glycerol (glycerine with a few drops of 5 N HCl added) and heated to detain the roots. Destained roots were then observed directly. Acidified glycerol was used as a floating carrier to observe the nematodes in root tissues under an inverted microscope.

Determination of rhizospheric microorganisms

Soil samples were taken 5-10 cm below the ground surface and placed in a sterile bag. The plate method was used to determine the bacterial concentration. Bacteria were cultured in an incubator at 24°C, and the dilution gradients for bacteria, actinomycetes, and fungi were 10^{-5}, 10^{-4}, and 10^{-3}, respectively. Three replicates were set for each dilution and averaged. Bacteria were cultured with beef extract peptone; actinomycetes were cultured with Gauze's synthetic medium No. one; fungi were cultured with Thayer-Martin culture medium. Colony growth was observed daily, and samples with 10 to 100 colonies/dish were counted in a timely manner. The mass of rhizospheric soil was determined by the drying process: 20 mL of soil suspension was placed into the evaporating dish to dry. The mass of the rhizospheric soils per milliliter of suspension was calculated. Finally, the number of microorganisms per gram of the dry rhizospheric soils was calculated.

Statistics and data analysis

The experiment was arranged in a randomized block design and performed in three replicates. Data were analyzed with Excel 2007 and DPS (Refine Information Tech, Hangzhou, Zhejiang, China) software. Data were statistically analyzed using analysis of variance (ANOVA) by SPSS 10.0. The least significant difference (LSD) was calculated for the significant data at the 0.05 or 0.01 significance levels.

RESULTS

Changes in the number of root knots in self-rooted plants of different tomato varieties after root-knot nematode inoculation

As shown in Table 1, there were no root knots in the roots of any of the three varieties of tomato before a root-knot nematode inoculation. After inoculation, roots of all three varieties were infected with root-knot nematodes. In particular, *spr2* had the largest number of root knots, 470 knots/plant, followed by WT at 330 knots/plant and *35S::PS* at 210 knots/plant. These data indicate a significant difference in resistance to root-knot nematode. Compared with plants with nor-sprayed MeJA, the number of root-knots decreased from 470 knots/plant to 230 knots/plant in *spr2* plants and from 330 knots/plant to 221 knots/plants in WT plants after post-inoculation spraying of MeJA. After nematode inoculation and foliar application of MeJA, the number of root knots in roots of *spr2* and WT plants significantly decreased compared with nor-treated plants, and it was similar to the number of root knots in *35S::PS* plants which were not treated with MeJA.It indicated that *35S::PS* plants' resistance to root-knot nematode was closely related to MeJA activity.

Changes in rhizospheric microbial composition of self-rooted tomato plants of different varieties after root-knot nematode inoculation

Changes in the rhizospheric microbial composition (Table 2) showed that WT plants had the largest number of total rhizospheric microorganisms (the soil dry sample) among different varieties of self-rooted tomato plants,

Table 1. Effects of different treatment on root-knot number in self-root tomato

Variety	Treatments		
	CK	N	N+M
WT	0	330Bb	221a
spr2	0	470Aa	230a
35S::PS	0	210Cc	-

Note: Data aremean value of six repetitions in the same treatment, the different capital and little letter mean differences from control at 0.01and 0.05 levels respectively. CK: no root-knot nematode N: root-knot nematode but no MeJA, N+M: root-knot nematode and MeJA.

while *spr2* plants had the smallest number. The total number of rhizospheric microorganisms, number of fungi (F), number of actinomycetes (A), number of bacteria (B), and the A/F ratio were significantly higer, while B/F was significantly lower, in *35S::PS* plants than in *spr2* plants. F, A and A/F were significantly higher, while B was lower, in self-rooted *35S::PS* plants than in WT plants. The number of microorganisms and B/F were significantly lower, while A/F was significantly higher, in *spr2* plants than in WT plants. The JA synthesis in JA-related mutant plants may have affected the number of rhizospheric microorganisms. A large number of JA can be rapidly synthesized in the wounded or intact part of the plant in a short time, which might affect the transport of underground rhizospheric material.

Table 2. Variation of main microorganism in rhizosphere soil of self-root tomato

Variety	Total quantity	Fungi (F) quantity /(10^6cfu·g^{-1}DM)		Actinomyces (A) quantity /(10^6cfu·g^{-1}DM)		Bacterium (B) quantity /(10^6cfu·g^{-1}DM)		A/F	B/F
		Number	%	Number	%	Number	%		
WT	773.98Aa	11.68Aa	1.51%	49.50Bb	6.40%	712.80Aa	92.10%	4.24Bc	61.02Aa
spr2	377.39Cc	7.13Bb	1.89%	43.56Bc	11.54%	326.70Cc	86.57%	6.11Bb	45.83Bb
35S::PS	561.13Bb	12.67Aa	2.26%	132.66Aa	23.64%	415.80Bb	74.10%	10.47Aa	32.81Cc

Note: The different capital and little letter mean differences from control at 0.01and 0.05 levels respectively.

Changes in rhizospheric microbial composition of tomato plants grafted to different rootstocks after root-knot nematode inoculation

Table 3 reports the changes in microorganisms cultured from soil samples that were taken 24 h after the root-knot nematode inoculation. It shows the total number of rhizospheric microorganisms (dry soil samples) in tomato plants that were grafted with different rootstocks: the total number was significantly higher in graft of WT/*spr2* andWT/*35S::PS*, than in the control (WT/WT). In particular, the numbers of both soil fungi and bacteria were significantly higher inWT/*spr2* than in WT/WT, while the number of actinomycetes was only slightly (not significantly) greater in WT/*spr2* than in WT/WT. The numbers of soil fungi, actinomycetes, and bacteria were all significantly higher in WT/*35S::PS*, than in WT/WT. In addition, the number of soil actinomycetes was significantly higher in WT/*35S::PS*, than in WT/*spr2*, while the number of fungi and bacteria were significantly smaller in WT/*35S::PS*, than in WT/*spr2*.

The changes in microorganisms cultured from soil samples that were taken 144 h after the root-knot nematode inoculation were showed in Table 4. The total number of rhizospheric microorganisms (dry soil samples) in tomato plants that were grafted onto different rootstocks: WT/*spr2* and WT/*35S::PS*, showed an increase in the

Table 3. Variation of main microorganism in rhizosphere soil of grafted tomato (24 hours)

Treatments	Total quantity	Fungi (F) quantity /(10^6cfu· g^{-1}DM)		Actinomyces (A) quantity /(10^6cfu·g^{-1}DM)		Bacterium (B) quantity /(10^6cfu·g^{-1}DM)	
		Number	%	Number	%	Number	%
(WT/WT)	442.43Bb	3.86Bb	0.87%	62.37Bc	14.10%	376.20Bb	85.03%
(WT/*spr2*)	610.53Aa	12.57Aa	2.06%	83.16Bb	13.62%	514.80Aa	84.32%
(WT/*35S::PS*)	610.04Aa	11.09Aa	1.82%	123.75Aa	20.29%	475.20Ab	77.90%

Note: The different capital and little letter mean differences from control at 0.01and 0.05 levels respectively.

total number compared with the control (WT/WT). In particular, the numbers of soil fungi and actinomycetes were significantly smaller in WT/*spr2* than in the control, while the number of bacteria was significantly higher in WT/*spr2* than in WT/WT. The numbers of soil fungi, actinomycetes, and bacteria were significantly greater in WT/*35S::PS*, than in WT/WT. The numbers of soil actinomycetes and fungi were significantly higher in WT/*35S::PS*, than in WT/*spr2*. In addition, for WT/WTplants, the total number of microorganisms was higher in soil samples taken 144 h after the nematode inoculation than in those taken 24 h after inoculation, possibly due to longer root-knot nematode infection.

Table 4. Variation of main microorganism in rhizosphere soil of grafted tomato (144 hours)

Treatments	Total quantity	Fungi (F) quantity /(10^6cfu· g^{-1}DM)		Actinomyces (A) quantity /(10^6cfu·g^{-1}DM)		Bacterium (B) quantity /(10^6cfu·g^{-1}DM)	
		Number	%	Number	%	Number	%
(WT/WT)	73.85Cc	14.45Bb	19.57%	9.90Bb	13.40%	49.50Bc	67.02%
(WT/*spr2*)	123.06Aa	6.24Cc	5.07%	7.92Bc	6.44%	108.90Aa	88.50%
(WT/*35S::PS*)	98.11Bb	26.83Aa	27.35%	11.88Aa	12.11%	59.40Bb	60.54%

Note: The different capital and little letter mean differences from control at 0.01and 0.05 levels respectively.

DISCUSSION

Plant resistance to soil-borne diseases is closely related to rhizospheric microorganisms (Pilkiewicz *et al*., 2008). Disease is usually significantly less severe in microorganism-rich soils than in microorganism-poor soils. The cotton plant's resistance to Verticillium wilt is positively correlated with the number of rhizospheric fungi and actinomycetes, negatively correlated with the number of nematodes, and not significantly correlated with the number of rhizospheric bacteria.The disease-resistant varieties have a greater number of rhizospheric microorganisms than the susceptible varieties, and the floristic composition is also more complex. Severely diseased plants of the susceptible varieties are low in rhizospheric antagonistic bacterial community diversity, while mildly diseased plants of the susceptible varieties and plants of disease-resistant varieties without disease show more rhizospheric antagonistic strains in screening (Ülbegi-Mohyla *et al*., 2009; Curtis *et al*., 2010). In this study, plants of all three varieties were infected with root-knot nematodes after inoculation; *spr2* plants had the most root knots, followed by WT plants and *35S::PS* plants. The total number of rhizospheric microorganisms, F, A, B and A/F were significantly higher, while B/F was significantly lower, in self-rooted *35S::PS* plants than in *spr2* plants. This result might be related to the synthesis of JA: in JA-related tomato mutant plants, JA might affect the number of rhizospheric microorganisms and thus generate antagonistic effects to root-knot nematodes, affecting the resistance of roots to root-knot nematodes. This possibility is consistent with previous studies. When plants were attacked, JA production increased in plant tissues leading to local and systemic induction of different classes of defensive compounds, including alkaloids, phenolics, flavonoid and various terpenoids (Shakil*et al*., 2005). Treatment of plants with exogenous application of JA presumably activated the natural defensive response of the plants and thus, enhanced the resistance to the attack. Exogenous JA and salicylic acid have preventive effects against root-knot nematodes and could significantly inhibit the formation of root knots and egg masses. Cooper *et al*. have shown that JA can induce systemically acquired resistance to root-knot nematodes in tomato plants (Cooper *et al*., 2005). Irrigation treatment with exogenous JA can induce resistance to nematodes in spinach and oat plants (Soriano *et al*., 2004).

Grafting may alter the number and composition of plant root exudates, thereby affecting the rhizospheric microbial flora composition and efficiently improving the resistance of plants to adversity. Grafting increased the number of rhizospheric nitrifying bacteria and

nitrogen-fixing bacteria. It indicated that grafting can increase the number of total rhizospheric bacteria and the physiological groups of bacteria in grafted eggplants. Finally it improved the condition of physiological groups of soil microorganisms (Wang *et al*., 2010). Jiang *et al*. found that the amount of rhizospheric bacteria, fungi, and actinomycetes in grafted pepper plants was significantly increased compared with the control.The ratio of actinomycetes to the total microorganisms was increased, while the ratio of bacteria and fungi to the total microorganisms was decreased. It indicated that the increase of the total microorganisms and actinomycetes was one of the main reasons for its enhanced resistance to root rot (Jiang *et al*., 2010). The results of this study showed that the numbers of soil fungi, bacteria, and actinomycetes were higher in grafted plants than in the control (WT/WT) after the root-knot nematode inoculation for 24 h. Using 35s and *spr2* plants as rootstocks seemed to improve the soil conditions to varying degrees, indicating that the root exudates of rootstocks not only exhibited allelopathic effects but also induced antagonistic effects against root-knot nematodes by affecting the structure of rhizospheric microbial populations. Meanwhile, the number of soil actinomycetes was significantly greater in WT/*35S::PS* than in WT/*spr2*, regardless of the time since the root-knot nematode inoculation for 24 h or 144 h. Actinomycetes are involved in the decomposition of soil organic matter, and the antibiotics they secrete can also inhibit the growth of other harmful pathogens (Kutluhan *et al*., 2011; Fazili *et al*., 2012). The decreased numbers of soil bacteria and actinomycetes and increased fungal composition indicate that the soils were becoming fungus-dominated. Fungal dominance is an important indicator of declining soil quality (Chagnon *et al*., 2013). In this study, the presence of a significantly greater number of soil actinomycetes may have been a major reason that WT/*35S::PS* had stronger resistance to root-knot nematodes than WT/*spr2*. The biggest difference between WT/*35S::PS* and WT/*spr2* was the JA synthesis in rootstocks. The former synthesized a large number of JA, while the latter did not. These facts show that changes in JA play an important role in tomato plants' resistance to root-knot nematodes during grafting. The data in this study suggest that the underlying causes of the improvement in resistance to root-knot nematode from changing roots by grafting is the induced synthesis of a large number of JA, which would affect rhizospheric microbial communities and, ultimately, induce resistance. In addition, although the microorganisms that could be cultured in this study could reflect the stable microbial community composition to some extent, their number represented only 0.01% to 10% of the total number of microorganisms. Therefore, in the future, new molecular biology techniques should be used to study grafted tomato plants' rhizospheric soil microorganisms that could not be cultured to investigate the effect of grafting on the structure and functional diversity of tomato plants' soil microbial community.

In summary, the grafting and jasmonic acid synthesis changed the rhizospheric microorganism amount and composition of tomato, increased the quantity of beneficial microorganism, thereby improve the resistance to root knot nematode. In order to declare the mechanism by which endogenous JA induces resistance to root-knot nematode, it would analyse the related gene and protein expression and their relationship in further.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (31171952), the Funding Project for Great Wall Scholar (CIT&TCD20130323), National Spark Program (2012GA600002). It was also financially supported by the Education Commission of Beijing (PXM2013_014207_ 0000_73, PXM2014_014207_ 000028), the Scientific and Technological Research Project of Beijing(D131100000713001), the 2014 Education Teaching-Research Training for Undergraduate Students from the Education Commission of Beijing.

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