

## Changes in Phytoplasma Densities in Witches' Broom-Infected Jujube Trees over Seasons

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The relative density of phytoplasmas in witches' broom (WB)-infected jujube trees was investigated using competitive polymerase chain reaction (PCR). During dormant and defoliating seasons, the densities of phytoplasmas were about the same in roots and twigs. In early growing season, the density showed the highest rates in roots, then in twigs and in petioles. However, the density was highest in petioles and the lowest in roots during actively growing season. Throughout the year, root samples did not show any serious fluctuation compared with that of twigs and petioles. Density was lowest during actively growing season in root samples. In contrast, petiole sample densities varied to a great extent depending on the season, very high during actively growing season, but very low during the early growing season. In twig samples, the densities were very high and almost the same in both defoliating and dormant seasons. Among the parts of the trees, phytoplasma density was the most stable in root samples throughout the year. The highest densities of phytoplasmas were about the same in all tree parts. These results suggest that the phytoplasmas may overwinter not only in roots but also in twigs, and that multiplication rate of phytoplasma becomes very high right after the early growing season.

**Keywords :** competitive PCR, density, phytoplasma, witches' broom, *Zizyphus jujuba*.

Witches' broom of jujube (*Zizyphus jujuba*) caused by *Phytoplasma* sp. is one of the most serious diseases of tree in Korea. It was first observed in middle part of Korea in the 1930s, and spread throughout the country by the 1950s (Kim, 1965). From that time on, the disease has threatened the cultivation of jujube, causing tree infertility. At present, jujube witches' broom (JWB) disease can be seen in many parts of the country. Jujube trees usually die within several years from the first appearance of witches' broom symptoms (Park et al., 1995).

Because the disease has been a major limiting factor in jujube cultivation, researches have been focused on the

development of control methods. As a result, JWB can now be successfully suppressed by using trunk injection of tetracycline antibiotics (Cha et al., 1992). However, the effect of antibiotics do not last long, and witches' broom (WB) symptoms reappear during the next season. If the time and method of antibiotics injection are not right, the effect of antibiotics will not last long enough (Cha and Tattar, 1993). Therefore, information about the distribution and density of phytoplasmas in the tree is important in making decision regarding antibiotics injection.

There have been several reports on the distribution and population fluctuation of phytoplasmas in the tree (Cha, 1998; Cha and Tattar, 1991; Seemüller, 1988; Tsai, 1988). Conventional researches used electron microscopy or fluorescence microscopy in the detection of phytoplasmas in the tissue. These detection methods, however, are not quite accurate or precise, and make false-negative or false-positive results very often. Fortunately, the new technique to detect nucleic acid, polymerase chain reaction (PCR), has been introduced as a new technique to detect nucleic acid, making it possible to detect very small amount of specific nucleic acid. Moreover, the new, improved PCR method (competitive PCR) was developed and applied in plant bacteriology a few years ago (Hu et al., 1995). Competitive PCR enables the quantitative analysis of microorganisms.

In this study, the distribution and population fluctuation of phytoplasmas were examined by using general and competitive PCR techniques in three kinds of witches' broom-infected jujube tissues over one year.

### Materials and Methods

**Plant materials.** JWB tissues used in this study were collected from an orchard in the suburb of Cheongju city, Korea. Jujube trees for this experiment were kept without any treatment during the period. Roots of about 5 mm thick, 1~2-year-old twigs, and petioles of jujube trees which showed WB symptoms were collected four times during one year: early growing, actively growing, defoliating, and dormant seasons. All tissues collected were kept in a deep freezer at -60°C for further use.

**Total DNA extraction.** The whole tissues of petiole except mesophyll and leaf vein, phloem tissues without outer bark and

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**Table 1.** PCR protocol for amplification of jujube witches' broom phytoplasma DNA

Component	Reaction mixture for detection	Reaction mixture for density fluctuation	Reaction mixture for nested PCR	
			First PCR	Second PCR
Total DNA solution	1.0	2.0	1.0	1.0
10× PCR buffer	3.0	3.0	3.0	3.0
25 mM MgCl <sub>2</sub>	1.0	1.0	1.2	1.0
10 mM dNTP	0.6	0.6	0.6	0.6
Forward primer (10 pM/μl)	3.0	3.0	3.0	3.0
Reverse primer (10 pM/μl)	3.0	3.0	3.0	3.0
Polymerase(5 U/μl)	0.2	0.2	0.2	0.2
Competitive DNA	–	1.0	–	–
Sterile distilled water	18.2	17.2	18.0	18.2
Total volume (μl)	30	30	30	30

xylem, and phloem tissues of roots without cortex and xylem, were used for petiole, twig, and root, respectively. Tissues of each part (0.2 g) were homogenized in liquid nitrogen with separate mortars and pestles. Total DNA was extracted following Namba et al. (1993). Extracted DNA was re-dissolved with 150 μl of sterilized distilled water and stored at -20°C until use.

**Amplification and detection of phytoplasma DNA.** Universal primer pairs described by Ahrens and Seemüller (1992) for phytoplasmas were used in the PCR and the sequences were 5'-ACGAAAGCGTGGGGAGCAAAA-3' for forward and 5'-GAAGTCGAGTTGCAGACTTC-3' for reverse. PCR method was modified from Ahrens and Seemüller (1992), where 30 μl of reaction mixture (Table 1) were placed into 0.2 ml of PCR microtube (Axygen; MCT-175-C™) and ran in an automatic PCR machine (Bio-Rad; Gene cycler™). After initial denaturation for 10 minutes at 94°C, 30 cycles of amplification were carried out. Duration and temperature of thermal cycle for PCR were 1-minute denaturation at 94°C, 2-minute annealing at 58°C, and 3-minute extension at 72°C. As exceptions, duration of both the first denaturation and the last extension were 10 minutes each.

PCR products were electrophoresed in 2% agarose gel (0.5X TBE) and stained with ethidium bromide (final concentration 50 ng/ml). Then, the gel was destained for 30 minutes in distilled water. The amplified phytoplasma DNA was visualized under the UV light.

**Identification of PCR products.** PCR products of petioles, which have lots of chloroplasts, and PCR products of twigs and roots, which have little chloroplasts, were reacted with the restriction enzyme *AluI* for 2 h to compare the polymorphism of amplified DNA bands. After that, each reacted mixture was electrophoresed in 20% polyacrylamide gel to compare the polymorphism of the bands.

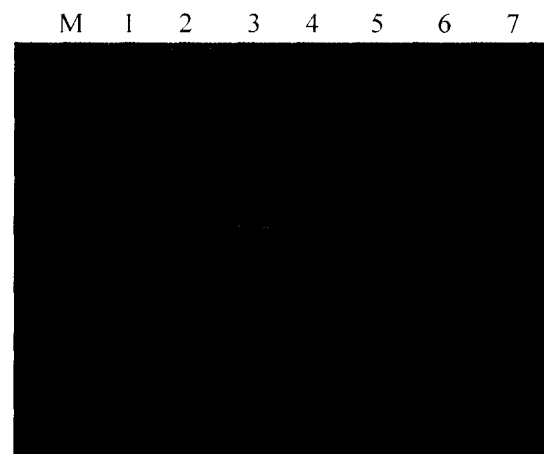
**Population fluctuation of phytoplasmas in the tissues.** To compare the population density of phytoplasmas in the tree tissues, competitive PCR technique was applied. The competitor DNA (340 bp) for competitive PCR was obtained by using the Competitive DNA Construction Kit™ (TaKaRa Co.). The concentration of competitor DNA ranged from 10<sup>7</sup> to 10<sup>2</sup> copies/μl by 10 times dilution method. Extraction of total DNA, primer pair for

PCR, and the conditions for PCR were exactly the same as the experiment for detection of phytoplasmas by PCR.

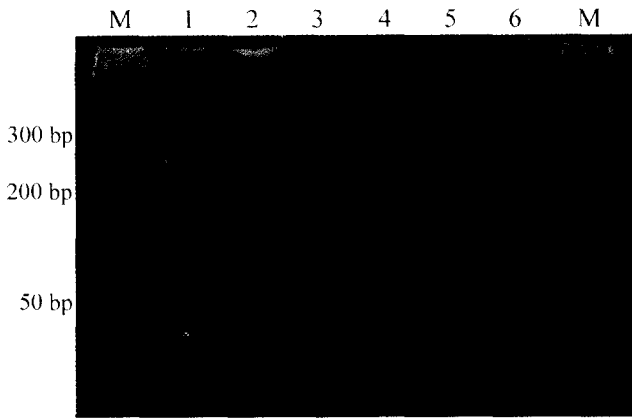
## Results

**Detection of phytoplasma DNA.** In this study, the target phytoplasma DNA was amplified from the samples of petioles, twigs, and roots of jujube tree that showed WB symptoms throughout the growing season. The size of the band was about 0.5 kb. Meanwhile, no band was detected from the total DNA samples of healthy looking young seedlings from jujube seeds (Fig. 1).

**Identification of PCR products.** In the experiment on restriction fragment length polymorphism using restriction enzyme *AluI*, the band patterns of amplified DNAs of petioles, twigs, and roots of witches' broom-infected jujube



**Fig. 1.** Agarose gel electrophoresis of PCR products amplified from petiole, twig, and root samples in witches' broom infected jujube (*Zizyphus jujuba*) trees. M, molecular weight marker; lane 1, petiole sample; lane 2, twig sample; lane 3, root sample; lanes 4-5, healthy tree samples; lanes 6-7, tap water.



**Fig. 2.** Polyacrylamide gel electrophoresis of *AluI* digests of PCR products obtained by primer pairs AS-1/AS-2. M, molecular weight marker; lanes 1-2, petiole samples; lane 3-4, twig samples; lane 5-6, root samples.

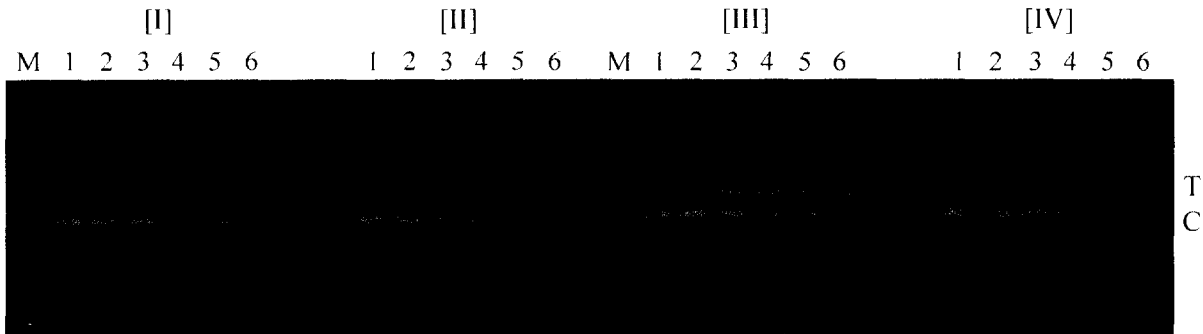
trees showed no polymorphism (Fig. 2). The results proved that the PCR products were the amplification of 16S rRNA gene of phytoplasma and not of chloroplast RNA.

**Population fluctuation of phytoplasmas in the tissues.** As a result of competitive PCR, the population density of

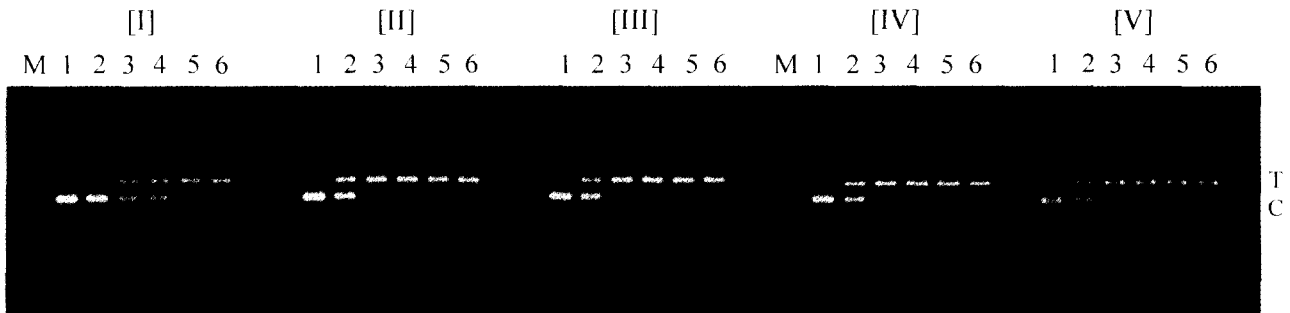
phytoplasmas varied depending on the sampling time and the part of the tissue. The population density of phytoplasmas was determined by the comparison of amplified competitor DNA and phytoplasma DNA. In the samples collected in June (early growing season), the relative density of phytoplasma DNA in petiole varied to a great extent and ranged from 1 to 10,000 copies per microliter (Fig. 3). The range of the phytoplasma DNA density was greater in twigs than in petioles. The lowest was 1 and the highest was 100,000 copies per microliter in twig samples (Fig. 4). On the other hand, all root samples contained 100,000 copies of phytoplasma DNA per microliter (Fig. 4). Therefore, the density of phytoplasma was in the order of roots, twigs, and then petioles, from the highest to the lowest.

During the actively growing season, the densities were relatively high (from  $10^3$  to  $10^6$  copies/ $\mu$ l) in petioles. Twig samples showed various densities, and ranged from  $10^1$  to  $10^5$  copies/ $\mu$ l. However, the density was very stable in root samples. The lowest was  $10^3$  but the highest also remained at  $10^4$  copies/ $\mu$ l. The density of phytoplasmas was quite high in this season regardless of the part of the tree, with petioles having the highest density, followed by roots.

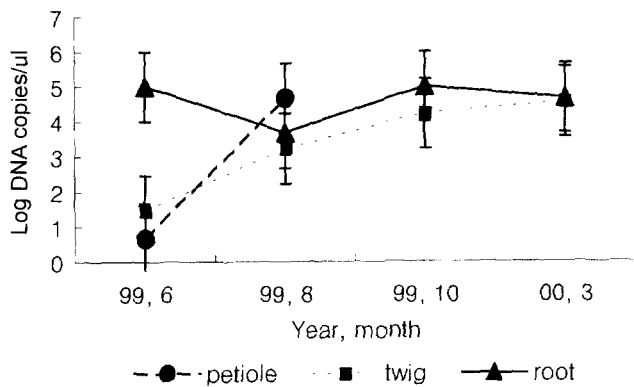
Petiole tissues were not tested during defoliating and dor-



**Fig. 3.** Agarose gel electrophoresis of PCR products amplified from petiole samples (I-IV) of witches' broom-infected jujube trees in early growing season. T: target DNA, C: DNA competitor. Lanes: competitor concentration (copies/ $\mu$ l) (1:  $10^7$ , 2:  $10^6$ , 3:  $10^5$ , 4:  $10^4$ , 5:  $10^3$ , 6:  $10^2$ ), M: molecular weight marker.



**Fig. 4.** Agarose gel electrophoresis of PCR products amplified from witches' broom-infected jujube twig samples (I-III) of defoliating season, and root samples (IV-V) of dormant season. T: target DNA, C: competitor DNA. Lanes: competitor concentration (copies/ $\mu$ l) (1:  $10^7$ , 2:  $10^6$ , 3:  $10^5$ , 4:  $10^4$ , 5:  $10^3$ , 6:  $10^2$ ), M: molecular weight marker.



**Fig. 5.** The population fluctuations of phytoplasmas in petioles, twigs, and roots of witches' broom-infected jujube trees over season (from June 1999 to March 2000).

mant season because the tree shed all the petioles and leaves down. The remaining parts of the tree, twigs and roots showed almost the same densities during the defoliating season. By the defoliating season, the densities of phytoplasmas of both twigs and roots increased to their highest (Fig. 5). In twig samples, the densities ranged from  $10^4$  to  $10^5$  copies/ $\mu$ l. However, all the densities were  $10^5$  copies/ $\mu$ l in root samples. It could be concluded that the phytoplasma density was almost the same in any part of the tree during the defoliation season.

The densities of twigs and roots during the dormant season showed the same pattern as that of defoliating season. Most twig and root samples revealed  $10^4$  to  $10^5$  copies of phytoplasmas per microliter of tissues.

Throughout the year, phytoplasma densities in the root were very stable (Fig. 5). On the contrary, twigs had high density of phytoplasmas from actively growing season to dormant season. The density was low during the early growing season. However, the phytoplasma density changed drastically in petioles, where it was observed only from the beginning of the growing season to defoliating season. Among the parts of the trees, phytoplasma density was the most stable in root samples throughout the year. The highest density of phytoplasmas was about the same in all the three parts of the tree.

## Discussion

When the PCR technique using phytoplasma specific primer pairs was applied to the detection of phytoplasma in tree tissues, specific DNA bands appeared from all the samples of WB infected jujube trees. On the other hand, no phytoplasma-specific fluorescence was detected from some of the samples (data not shown). Results revealed that the PCR technique is superior to the electron or fluorescence microscopy or serological methods in detection sensitivity,

at least in phytoplasma detection (Ahrens & Seemüller, 1992; Chen et al., 1994; Han et al., 2001; Schaff et al., 1992).

In disease diagnosis using PCR technique, the most important factors are the specificity of primer and the conditions for DNA amplification. There have been several reports that sometimes the contaminated DNAs of other sources, especially chloroplast DNA, were also amplified during PCR. At the beginning of these experiments, some DNAs were also amplified even in samples used as negative control (seedlings from seed) because of the unfavorable PCR conditions. Therefore, to confirm whether the amplified products obtained in the PCR were from phytoplasma DNA, the PCR products were subjected to restriction enzyme *AluI* was treatment following Ahrens and Seemüller (1992), who reported that 16S rRNA of plant chloroplasts could be amplified, and suggested the method to differentiate PCR products of chloroplast and phytoplasma. Therefore, the concentration of magnesium chloride in reaction mixture, annealing temperature, and the number of PCR cycle were adjusted many times in this experiment (data not shown). Finally, the most optimum PCR conditions for the differentiation of chloroplast DNA and phytoplasma DNA were established in this experiment. Digestion by restriction enzyme *AluI* for RFLP could be an indication that the products are of phytoplasma DNA.

Competitive PCR was developed to overcome the limit of general PCR such as false negative results caused by low specificity and sensitivity of primers, and various PCR inhibition substances in the plant tissues. Hu et al. (1995) reported that, in the density measurement experiments of the bacterium *Clavibacter michiganensis* subsp. *sepedonicus*, competitive PCR technique was more sensitive than direct colony counting method. If competitive PCR is used, the density of the pathogen could be measured without culture of the pathogen and measuring time could be shortened. Therefore, the competitive PCR technique is very useful in the detection and the density measurement of unculturable obligate parasites such as phytoplasmas.

The competitive PCR technique was also applied in these experiments, which revealed several interesting results about the distribution and the population fluctuation of phytoplasmas in jujube tissues. Among the parts of the tree, roots were the most colonized by phytoplasmas. Phytoplasma density of root samples was not lower than other parts of the tree, and the density was almost stable throughout the year. The density in the twig samples was almost stable throughout the year except during the early growing season. However, these results were not consistent with the results of Tasi et al. (1988). By electron microscopy, they reported that phytoplasmas were observed in

the underground tissues of coconut palm plant only from February to April, and from April to November for the aboveground parts. On the other hand, Cha and Tattar (1991), who were using fluorescence microscopy, reported that more than 80% of the root tissues of yellow-infected white ash trees showed positive fluorescence reaction. They also said that the positive fluorescence was clear from May to October in twigs and the fluorescence was very weak during the dormant season (from December to March). Seemüller (1988) also worked on the population dynamics of phytoplasmas using fluorescence microscopy. According to him, more than 80% of the tissues showed positive fluorescence reaction from August to February in stems and from August to June in roots. However, the rates of fluorescence in twig tissues were lower than 80% throughout the year except July. The results of these experiments were consistent with Seemüller's (1988) experiments in some aspects only. Cha and Tattar (1991), Seemüller (1988), and this study almost got the same results on root samples regardless of the tree species.

The differences in the research results can be attributed to several factors. Among these is the difference in the species of host plants and their growing environment. In addition, phytoplasma detection techniques must have affected the results as well. Between this study and past studies (Cha and Tattar, 1991; Han et al., 2001; Seemüller, 1988) the difference in the results may be due to the difference in the sensitivity of detection methods used in the experiments. Generally, fluorescence microscopy is known to be quite sensitive and a very simple method for phytoplasma detection. However, the most serious disadvantage of fluorescence is false negative reaction (Seemüller, 1976). Therefore, the low sensitivity and high rates of false negative result might have caused the difference in the result.

Competitive PCR was very useful in the research of population fluctuations of unculturable phytoplasmas in plant tissues. Following the methodology, the technique is not only relatively simple but the detection sensitivity is also very high. Results of this study on phytoplasma density in the trees over seasons suggest that the phytoplasmas may overwinter not only in roots but also in twigs, and multiplication rate of phytoplasmas becomes very high just after the early growing season. However, the decrease in density of phytoplasmas in twigs during spring and early summer is still not fully explained.

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