Preliminary Evaluation of Pistachio Leaf Chlorosis Etiology in Ardakan Planting Area

Mohammad Moradi (PhD)[1*], Seyed Reza Fani (PhD)[2], Hamid Alipour (PhD)[1], Abbas Farajpour (BS)[1], Hydar Masoumi (MSc)[1]

[1] *Pistachio Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rafsanjan, Iran*

[2] *Plant Protection Research Department, Yazd Agricultural and Natural Resources Research and Education Center (AREEO), Yazd, Iran*

Information

Article Type:
Original Article

Article History:

Received: 04.04.2024
Accepted: 11.06.2024

Doi: 10.22123/phj.2026.384485.1147

Keywords:
Yellowing
Disorder
Elemental treatment
Pathogen
Nematode

Corresponding Author:
Mohammad Moradi

Email: : moradi@pri.ir

Tel: +98- 3434225205

Abstract

Background: Over the past twenty years, yellow chlorosis has been appearing in various pistachio cultivars in the Ardakan region. In cases of severe infection, the trees may be completely destroyed. The development of leaf chlorosis can take several years before affecting the trees die.

Materials and Methods: Several experiments were carried out to assess the roles of fungi, nematodes, and phytoplasma as disease causes, along with the potential for pathogen transmission via grafting. Moreover, some trees displaying different levels of leaf chlorosis were trunk-injected with macro- and micro-elements.

Results: Based on evaluations of 500 bud grafts, the results indicated that less than 2% of the grafted sprouts exhibited symptoms of infection transmission after two years. All samples tested negative in PCR assays for the detection of plant phytoplasmas. Various isolates of fungal species, such as *Fusarium*, were identified from the roots of trees exhibiting chlorosis; however, Koch's postulates could not be confirmed. Additionally, several genera of nematodes, including Meloidogyne, *Pratylenchus*, *Helicotylenchus*, and *Tylenchulus*, were found in the rhizosphere of pistachio trees. Among these, the lesion nematode Pratylenchus neglectus was identified with a high frequency and was recognized as the most important and predominant species. Injections of macro and micro-elements into trees displaying different intensities of chlorosis alleviated the symptoms of the disease, with the effectiveness influenced by the timing of injection and the severity of the symptoms.

Conclusion: Pistachio chlorosis may be caused by a combination of biotic and abiotic factors, which require further investigations.

►Please cite this article as follows:
Moradi M, Fani SR, Alipour H, Farajpour A, Masoumi H. Preliminary Evaluation of Pistachio Leaf Chlorosis Etiology in Ardakan Planting Area. Pistachio and Health Journal. 2024;7(1-2):52-60.

1. Introduction

Yazd province is the third largest producer of pistachios in Iran [1]. The presence of old pistachio orchards as well as the creation of new orchards in Chah-Afzal, considering the genetic bank of pistachio cultivars in Ardakan. In the last decade, yellow chlorosis has been observed on different cultivars of pistachio trees. In severe infection, the tree will be destroyed. The onset of leaf chlorosis may take several years before the trees die. Different scenarios have been proposed causing chlorosis in the trees, such as poor drainage, root damage, alkalinity, nutrient deficiency, poor quality and quantity of irrigation, soil texture and structure, as well as environmental pollution. Imbalance of nutrients in the soil and their deficiency, high alkalinity or salinity of the soil, insufficient ventilation, and suffocation of roots due to excessive irrigation and too high soil moisture for a long time are the most important scenarios that may happen. Under the conditions of anaerobic respiration, acidity and accumulation of respiratory gases in the water solution, the life of microorganisms [2]. Yellowing or chlorosis can also be caused by insufficient plant access to iron or inactivation of iron in the plant. Of course, in most soils where the trees turn yellow, there is enough iron, but due to soil and irrigation conditions, the plant is chlorinated and not able to absorb enough iron, or iron is absorbed. It becomes unusable inside the tissues of trees [3]. Different plant pathogens can also cause chlorosis in trees, such as plant parasitic nematodes and unknown viral, bacterial, or Phytoplasma [4].

In a study in the village of Yolcati in Gaziantep, Turkey, in a pistachio orchard with a high amount of lime, symptoms were observed as yellowing of the leaves and the greenness of the veins. The use of ferrous sulfate with a concentration of 55 ppm with pH 3, three times, has been the most effective foliar treatment to reduce yellowing. In soil application of 4 to 6 kg of ferrous sulfate per tree has been observed to be effective [5]. Under flooding conditions, the roots of trees become weak, which makes them susceptible to secondary plant pathogens. On the other hand, high moisture conditions in the soil increase the population of nematodes, especially root wound nematodes, and various fungal diseases. Under these conditions, nutritional roots are impaired and absorption is poor, which may cause yellowing in tree shoots [6]. The effect of flood stress on soil depends on changes in oxidation status and soil regeneration. During long periods of flooding, the microbial flora changes in favor of anaerobic microorganisms that use an electron receptor other than O_2. Substances such as nitrates, quaternary manganese, ferric iron, sulfates, and finally sulfites are reduced by electron capture, resulting in the reduction and toxic forms of mineral ions. Anaerobic microbes are less prone to organic matter and have less effect than aerobic microorganisms. As a result, the decomposition rate of organic matter in flooded soils will be slower. Also, the final products produced by anaerobic decomposition are toxic to plants. Toxins produced during decomposition, especially during the first two weeks after decomposition, can reduce or stop plant growth. When the soil is in a reduction state, the concentrations of iron, aluminum, and magnesium increase in exchangeable sites, and some of the phosphorus in the soil becomes soluble and is exposed to leaching [7]. Studies have shown that ethylene accumulates in the soil when submerged, destroying and restricting plant growth. This reduces the absorption of soil

nutrients and leads to less biomass production. Light and frequent irrigations help to aerate the soil and are very effective in removing the yellowing of trees. The use of drip irrigation methods increases the permeability of the soil around the roots and prevents water saturation [8]. Different pathogens, directly and/or indirectly, can cause a variety of yellowing in plants, which can be attributed to plant pathogenic nematodes, especially lesion nematodes. On the other hand, some fungal vascular wilt, viruses, Phytoplasmas, and xylem-limited bacteria (XLB) can cause symptoms of systemic yellowing in the host plant. Many viruses and virus-like organisms are transmitted by insect vectors, including aphids, leafhoppers, or plant hoppers [4]. However, yellowing caused by plant pathogens in many cases may be different from yellowing caused by non-living factors. In many cases, the interaction of these pathogens with each other or in combination with abiotic stresses causes synergism of yellowing in trees [4]. The *Xylella fastidiosa*, which is limited to the xylem, causes symptoms of yellowing of the leaf margin (Scorch) in hosts such as grapes, almonds, plums, elms, and oaks. *X. fastidiosa* also causes dwarfism in peaches, alfalfa, and several weeds [9]. Due to the scarcity of information on the chlorosis of pistachio, the present study was conducted to determine the different possible causes of this disorder in Ardakan. In the older pistachio orchards across Ardakan, chlorosis was observed to varying degrees, with a notably higher prevalence in the pistachio farming regions of Ahmadabad. The Nili region exhibited the highest prevalence of chlorosis-affected trees, with 24.2% of trees showing symptoms. Moderate levels of chlorosis, ranging from 15% to 18%, were observed in the Ahmadabad, Faizabad, and Kamalabad regions. Among these areas, Ahmadabad stood out with the most severe consequences, as it recorded the highest percentage (2.1%) of trees that had completely dried out due to this condition (Unpublished data).

2. Materials and methods

2.1. Possibility of transmission of pathogens through grafting and leafhopper

In 2008, trees exhibiting various types and intensities of yellowing symptoms were selected across several orchards to study the potential for disease transmission. In June 2009, many grafts were prepared from symptomatic trees and transplanted onto healthy ones. Additionally, grafts from mature, healthy trees in Rafsanjan were grafted onto yellowing trees in Ardakan. Standard transplantation methods, such as bud and tubular grafting, were employed during spring and summer. The transplanted trees and seedlings were monitored for up to four years to assess whether the disease was transmitted pathogen.

2.2. Phytoplasma detection

Thirty branches and twigs of pistachio trees with a length of 5-10 cm with yellowing symptoms from different orchards in the Ardakan region were sampled and transferred to the laboratory in an ice container. Leaf veins were isolated from each sample, and 0.5 g of them was stored at -80 ° C for further experiments. Potato and lemon leaves with witches' broom disease and healthy plants without symptoms were also used for positive and negative control, respectively. DNA was extracted and purified by liquid nitrogen and the Fermentas DNA kit (Fermentas, Germany). The quality and quantity of purified DNA were measured by electrophoresis and

spectrophotometry, respectively. Finally, DNA with a concentration of 50 ng/µl was prepared to test for the presence of phytoplasma as a working solution [10]. Primers p1/p7, R16F2n/R16R2, and fU5/RU3 were used to evaluate the presence of phytoplasma [11]. PCR reactions in 25 µl volume including 19.15 µl of deionized water, 2 µl (5 ng) of genomic DNA template, 1 µl (20 µM) of each primer p1/p7 [12], 2.5 µl 10X PCR buffer, 1 µl MgCl2 (50 mM), 0.2 µl dNTP (100 mM) and 0.15 µL Taq DNA polymerase (5 U/µl) (Sinaclon, Iran) in a thermocycler (Primus, MWG Biotech, UK) as scheduled thermal including initial denaturalizing at 94 °C for 5 minutes, denaturalizing at 94 °C for 30 seconds, hybridization at 55 °C for 30 seconds, extension at 72 °C for 1.5 minutes in 30 cycles, and the final extension was performed at 72 °C for 7 minutes. For DNA staining, 2 µl of ethidium bromide (0.5mg/ml) was added. To observe the PCR product, electrophoresis with a constant voltage of 85 V for 1 hour was performed in 2% agarose gel (Sinaclon, Iran) in TAE buffer (Hydroxymethyl, 242 mg; Acetic acid, 57 ml; EDTA, 18.6; Water 1000 ml). It was photographed under UV light using Gel Documentation (UVtech, UK). To determine the weight of the fragments, a DNA size indicator called Gene RulerTM DNA ladder mix (Thermo Fisher, Massachusetts, USA) was used.

2.3. Isolation of fungal pathogens

Roots and branches of trees with chlorosis symptoms were sampled. 0.5 cm pieces were prepared and sterilized with 1% sodium hypochlorite and washed three times with sterile distilled water. After drying, the samples were cultured on potato dextrose agar (PDA, per L: 39 g potato dextrose agar, pH 6.5), corn meal agar with antibiotic (CMA-PARP, per L: 17 g corn meal agar, pimaricin solution 0.4 ml, ampicillin 0.25 g, rifampicin solution one ml, PCNB 5 ml) and Czapeck Dox agar (CDA). Fungal isolates obtained from plant tissues were stored in an agar slant tube. To implement Koch's postulate and ensure the pathogenicity of the isolates, a spore suspension at a concentration of 10^6 was prepared and was added around the 3 3-month-old seedlings. After inoculation, pistachio seedlings were first visited daily and then weekly to check for any symptoms [4].

2.4. Extracting nematodes from soil samples

To examine the role of pathogenic nematodes in chlorosis, samples of rhizosphere soil and roots from diseased trees were collected and brought to the laboratory. Nematodes were extracted from 250 g of soil using sieving and centrifugation techniques [13]. The extracted nematodes were detected with the plant nematode identification key [14].

2.5. Trunk injection of micronutrients and macronutrients into the trunks of diseased trees

Forty trees exhibiting various types and intensities of chlorosis were selected from two orchards in Ahmadabad and one orchard in Asilabad. In April of 2011 and 2012, these trees were injected with micronutrients and macronutrients using the following method. A hole was drilled downward at a 45-degree angle, 30 cm above the ground, using a 6 mm drill. An injector nozzle was then securely attached, and the nutrients were injected under pressure into the vascular system of the affected trees. The amount of fertilizer injected was determined based on the tree's characteristics; however, as a general guideline, approximately 1 to 2 ml of solution per centimeter of trunk diameter (at the

injection site) was administered. The results were evaluated monthly over the two years following the injections.

3. Results

3.1. Distribution of chlorosis in pistachio growing areas of Ardakan

Chlorosis was observed in most pistachio orchards of Ardakan, although with different frequencies. Most tree chlorosis was observed in Nili, Asilabad, Kamalabad, Feyzabad, and Majdabad. In the infected orchards, newly planted seedlings also died of chlorosis after a few years (Figure 1).

Fig 1. Different symptoms of pistachio leaf chlorosis and progressive necrosis

3.2. Possibility of chlorosis transmission by grafting

Based on evaluations of 500 bud grafts, the results indicated that less than 2% of the grafted sprouts exhibited symptoms of infection transmission after two years. Further observations revealed that chlorosis symptoms were transferred to the main body of the transplanted trees. In two cases, tree mortality was observed after five years. This issue highlights the various types of chlorosis that may be present in the Ardakan region, as well as the influence of other factors on the yellowing of pistachio trees.

3.2. Detection of Phytoplasma in trees with chlorosis

In all samples, the results of using the PCR method for the detection of plant phytoplasmas were negative, which eliminates the role of plant phytoplasmas in the development of chlorosis in the Ardakan area (Figure 2).

Fig 2. Electrophoresis of PCR products obtained from DNA purified from trees with chlorosis in Ardakan planting areas in comparison with in samples infected with plant phytoplasma from other pistachio growing areas: rows 1 to 26 of trees with chlorosis (R16F2n/R16R2 primer), rows 27 to 30 samples from Khorasan Razavi province, + positive control, -p negative control of pistachio tree without chlorosis, M, a 250 bp marker

3.3. Fungal isolates

Different isolates of fungal species, such as *Fusarium*, have been isolated from the roots of chlorosis trees. Inoculum production of these isolates and their inoculations did not produce disease on pistachio seedlings under greenhouse conditions after 6 months. Due to the fact that the above isolates were not able to cause disease on pistachio seedlings, they were not subjected to identification. The results of culturing branch samples with symptoms similar to *Verticillium* wilt on the different culture media also no pathogenic fungi were isolated. Some bacterial isolates have been isolated from *Verticillium*-like discoloration symptoms, which required further investigations.

4.3. Plant parasitic nematodes

Numerous nematodes from the rhizosphere of pistachio trees were identified with chlorosis symptoms that were both plant parasitic and non-parasitic. However, the lesion nematode *Pratylenchus neglectus* was identified with high frequency and as the most important species. No male was observed among *P. neglectus* populations. The characteristics of the female nematode were as follows. Wormy nematodes with a relatively strong cylindrical body that bends to the abdomen. The head is short, flush with the body, has two rings, the length of the rings is often the same, the height of the head is 2.5 to 3 and its width is 8 to 8.5 µm. Strong cuticle network of head and stylet, conical section half the length of the stylet or less. The style knots were distinct, spherical, and oblique. The distance between the dorsal esophageal gland and the stylet node was 3 to 4 µm. The expanded esophagus, the primary esophageal tube, is wide and has a certain compression at the junction with the esophageal median bulb. The median bulb is almost ovoid, and the esophageal glands cover the abdomen at the beginning of the intestine. The lateral surfaces have four lines that extend to the end of the tail. Body surface lines were defined. The vagina is slit shape and closed, the ovary was forward, the eggs were in a row, the sperm storage bag was round, quadrangular, empty of sperm, and hardly visible. The posterior pouch of the uterus was 1 to 1.6 times the width of the body, the tail was cylindrical, and the end was conical, round, and tended to stick to the bed diagonally and flat. In general, the tail has various changes in different nematodes. The phasmid is located in the first half of the tail (Figure 3).

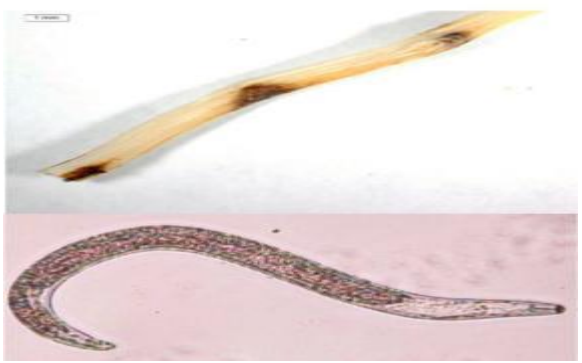

Fig 3. Symptoms of root wound nematode damage on the secondary root of the pistachio tree and the juvenile stage 2

5.3. Injection of the trunks of diseased trees

Injection of trees with different intensities of chlorosis relieved the symptoms of disease. This was influenced by the time of injection and the severity of the symptoms. Most of the injected trees responded positively and showed no signs of chlorosis up to 2 years. Injection after leaf emergence in trees caused phytotoxicity, which could cause death in trees that were severely affected. However, the injection had the best effect before or at the time of bud swelling and improved the chlorosis of the trees. This was well observed in dying trees. Injection of trees at the onset of chlorosis delayed the symptoms for at least 3 years (Figure 4).

Fig 4. Recovery of symptoms of pistachio leaf chlorosis after mineral nutrition via trunk injection

4. Discussion

Pistachio chlorosis in Ardakan appears to be a complex, largely non-pathogenic phenomenon, as only 2% is graft-transmissible. Seedlings planted in chlorotic orchard soil remained asymptomatic for two years, and symptomatic trees tested negative for phytoplasmas. Furthermore, nutrient injections failed to resolve the chlorosis, suggesting abiotic factors. Advanced techniques such as analytical and PCR tools, and electron microscopy are required to fully elucidate the primary causes of this chlorosis, which conventional methods may not be sufficient to uncover. Grafting experiments showed that only 2% of scions from chlorotic trees induced chlorosis in healthy trees. Scions from healthy trees grafted onto chlorotic trees developed similar symptoms, suggesting chlorosis may be pathogenic or non-pathogenic. While *Fusarium* species were isolated from decaying root samples, their pathogenicity remains unproven. Root knot nematodes (*Meloidogyne* sp.) were also observed. Further study is needed to understand the interaction of *Fusarium* with root lesion and root knot nematodes in situ, as this interaction may contribute to root rot, particularly in heavily irrigated soils. However, the relationship between these pathogens and chlorosis is unclear due to nutrient injections and the trees' positive response to them. Root lesion nematodes were the dominant nematode population in most samples. When present, other plant-parasitic nematodes occurred at very low levels. While root lesion nematode damage is typically assessed by nematode count per 100g of soil, the damage level varies based on nematode species, climate, soil type, and host plant. An accepted economic damage threshold is one to two nematodes per gram of soil, though this can range from 50 to 1800 nematodes per 100g of

soil [15]. The population of nematodes in the soil is a function of soil moisture [16]. Soil moisture studies revealed high soil moisture levels in Ardakan pistachio orchards, resulting in a high nematode population (more than 50 per 100 grams of rhizosphere soil), which necessitates management. Also, high soil moisture in many cases leads to an increase in the population of fungi, which can cause disease in roots weakened by abiotic stress.

5. Conclusion

The present study indicates that pistachio chlorosis may be caused by various agents, including both biotic and abiotic factors. The possibility of transmission through grafting suggests the presence of pathogens that are limited to the xylem and phloem. However, no evidence was found to suggest that phytoplasma or fungi are directly responsible for the chlorosis observed in Ardakan. Further investigation into pistachio chlorosis is needed, utilizing advanced molecular, biochemical, and analytical tools.

Acknowledgments

The authors are thankful to the Iranian Pistachio Center (IPC) for providing the required materials, facilities and for assisting with this project.

Funding

The authors thank the Office of Ardakan governor and Jihad-Agriculture management for their financial support.

Conflict of interest

The authors state that there is no conflict of interest.

Code of ethics

In this research, no living entity has been used, and the research stages have been conducted in a laboratory.

References

1- Ahmadi K, Ebadzadeh HR, Hatami F, Hosseinpour R, Abdeshah H. Agricultural statistics of Iran. Information and Communication Technology Center, Planing and Economic Affair, Ministry of Agriculture-Jahad. **2017**;3:*239.*

2- Liao CT, Lin CH. Physiological adaptation of crop plants to flooding stress. In Proceedings of the National Science Council, Republic of China. B, Life Sci. **2001**;25:*148-57.*

3- Malakouti MJ, Tabatabayi SJ. Proper nutrition of fruit trees. Iranian Soil and Water Institute Publication; **1999**.

4- Agrios GN. Plant pathology. Elsevier. **2005.**

5- Tekin H, Arpaci S, Yukceken Y, Cakir I. Pistachio nut iron deficiencies on calcareous soils. In: II International Symposium on Pistachios and Almonds; 1997 Aug 24; Tehran, Iran. Acta Horticulturae; **1997**. *p. 421-428.*

6- Schnürer J, Clarholm M, Boström S, Rosswall T. Effects of moisture on soil microorganisms and nematodes: a field experiment. Microb Ecol. **1986**; 12:*217-230*.

7- Visser EJ, Voesenek LA, Vartapetian BB, Jackson M. Flooding and plant growth. Ann Bot. **2003**;91:*107-9.*

8- Jackson MB, Ishizawa K, Ito O. Evolution and mechanisms of plant tolerance to flooding stress. Ann Bot. **2009**;103:*137-42*.

9- Purcell,AH, Hopkins DL. Fastidious xylem-limited bacteria plant pathogens. Annu Rev Phytopathol. **1996**;34:*131-151.*

10- Khodaygan P, Moslemkhani C, Shahbazi R, Moradi M, Habibi H. Molecular identification of 16S rII phytoplasma group in commercial pistachio cultivars in Iran. Arch Phytopathol Plant Prot. **2014**;47:*1400-07*.
11- Gundersen DE, Lee IM. Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathol Mediterr. **1996**;35:*144-51.*
12- Deng S, Hiruki C. Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J Microbiol Methods. **1991**;14:*53-61*.
13- Dropkin VH. Introduction to plant nematology. New York (NY): John Wiley & Sons; **1980**.
14- Mai W. Plant-parasitic nematodes: a pictorial key to genera. Ithaca (NY): Cornell University Press; **2018**.
15- Potter JW, Olthof THA. Nematode pests of vegetable crops. In: Evans K, Trudgill DL, Webster JM. Eds. Plant Parasitic Nematodes in Temperate Agriculture. CAB International. **1993**. *p. 171-207.*
16- Schnürer J, Clarholm M, Bostrbm S, Rosswall T. Effects of Moisture on Soil Microorganisms and Nematodes: A Field Experiment. Microb Ecol. **1986**;12:*217-230.*