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Research Article

Resistance response to *Clavibacter michiganensis* subsp. *michiganensis* in different available tomato cultivars in Iran

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Abstract: Tomato bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is a destructive tomato disease in the world and Iran, which can seriously affect the quality of the product. An integrated management program using pathogen-free seeds and resistant cultivars is necessary for disease control. In this study, the reaction of 24 cultivars of tomato was assessed against the disease under greenhouse conditions. Inoculation of seedlings at the 4-5 leaf stage was carried out by injecting a bacterial suspension of 1×10^4 CFU ml^{-1} at the axil, where the petiole meets the stem. The response of cultivars to the disease was evaluated via three indices, including the time of disease onset, disease severity (DS), and the area under the disease progress curve (AUDPC). The results indicated that AUDPC positively correlated with the time of disease onset (r = 0.85) and disease severity (r = 0.86). Based on the current findings, applying different indexes in response of tomato cultivars to bacterial canker disease provides accurate information about host resistance. The AUDPC index is the most reliable as it has a high positive correlation coefficient with two other indexes. In this study, cultivars 'Rio Grenade', 'King Stone', 'Early Urbana Y', 'CalJ N3', and 'Hyb. Firenze' showed more resistance against the disease than other studied cultivars. Usage of the mentioned cultivars is recommended in the integrated management program of tomato bacterial canker disease.

Keywords: AUDPC, bacterial canker, vascular infection, disease resistance

Introduction

Tomato *Solanum lycopersicum* L. is one of the most consumed vegetables in the Solanaceae family and is native to South and Central America (Bergougnoux, 2014). According to the Food and Agriculture Organization of the United Nations (FAO), the area under tomato cultivation is 5,051,983 hectares, and its production is more than 186 million tons worldwide (FAOSTAT, 2022). Several infectious and non-infectious factors can affect and limit the production of this product. More than 60 pathogens can attack tomatoes and cause several problems in yield and production. The most devastating bacterial diseases of tomatoes are bacterial speck, bacterial leaf spot, bacterial canker, bacterial wilting, and soft rot (Jones *et al.*, 2014).

Bacterial canker is a systemic vascular disease of tomato caused by *Clavibacter michiganensis*

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subsp. *michiganensis* (Cmm) (Jang *et al.*, 2022). For the first time, the disease was reported and described by Smith in the early 20th century from a greenhouse in the state of Michigan, US, and now is a severe disease in most tomato-growing areas around the world (Borkar and Yumlembam, 2017; Peritore-Galve *et al.*, 2021). The disease caused extensive epidemics in 1930-1980 in the United States and Canada. Tomato plants are grown from seed, and the epidemics of this disease occur due to disease transmission via infected seeds; therefore, high-quality and pathogen-free seeds are essential to produce healthy plants (Sen *et al.*, 2015).

The economic damage of this disease varies under farms or greenhouse conditions. A decreased yield of 84% was reported in commercial tomato farms in Ontario, Canada, while the rate of disease damage in artificially inoculated plants was variable from 46% to 93% (Nandi *et al.*, 2018). The primary host of Cmm is tomato; however, the disease is reported in some other crops and weeds of Solanaceae (Sen *et al.*, 2015).

The first observation of bacterial canker disease in Iran was in 1988, in tomato farms around Urmia, North West of Iran. Since then, the disease has been observed and reported in different tomato cultivation regions of the country (Osdaghi *et al.*, 2018).

Infected tomato plants show different symptoms depending on the sensitivity of tomato cultivars, degree of bacterium virulence, time and type of infection (systemic or local), and environmental conditions, especially temperature and humidity (de León *et al.*, 2011; Nandi *et al.*, 2018). Disease symptoms include leaf margin necrosis, browning of vascular tissue, unilateral wilting, dwarfing, and stem canker. (Boyaci *et al.*, 2021). Small and brown lesions on the fruits are called bird's eye spots surrounded by a white halo (Tancos *et al.*, 2013). Cmm can colonize the tomato plant for several generations without symptoms (Nandi *et al.*, 2018).

An intensive integrated management program by various methods such as rotation with non-host plants for three to four years, weed control, usage of Cmm-free seed and seed disinfection with chemical compound and thermotherapy, removal of plant debris to prevent pathogen spread, and use of copper compounds is essential to prevent economic severe losses (Sen *et al.*, 2015). It should be noted that none of the mentioned methods provides complete disease control and only plays a role in reducing pathogen spread (Boyaci *et al.*, 2021; Yokotani *et al.*, 2021). Using biological control agents might be an alternative procedure to disease control (Jang *et al.*, 2022). However, this method has not been successful in the field until now (Mohd Nadzir *et al.*, 2019; Karthika *et al.*, 2020).

Development of resistant cultivars is usually difficult, because of the high diversity in bacterial strain pathogenicity, various disease symptoms, differences in disease incidence at plant growth stages, and the effect of environmental conditions on disease progression (Yang and Francis, 2007). No commercially successful Cmm-resistant tomato cultivars are available (Sanver *et al.*, 2022). However, developing resistant or tolerant cultivars has been conducted for decades in several countries (Wang *et al.*, 2018). Although bacterial canker has caused a severe loss in tomato production worldwide, progress in breeding to achieve tolerant or resistant cultivars is slow (Yuqing *et al.*, 2018).

The germplasm, Hawaii 7998, is reported to be tolerant to the leaf phase of disease or local infection but susceptible to systemic contamination (Yang and Francis, 2007). Resistance against Cmm has been observed in some wild relatives of tomato, such as *Lycopersicon hirsutum*, *L. peruvianum*, *L. pimpinellifolium*, *L. chilense*, and *Solanum habrochaites* (Yang and Francis, 2007; Abebe *et al.*, 2022). Moreover, seven tomato accessions from species of *Solanum arcanum*, *S. habrochaites*, *S. pennellii*, and *S. peruvianum* were screened against the highly virulent Cmm strain in Turkey and based on the results, two accessions including *S. habrochaites* LA1777 and *S. arcanum* LA2157 were found to be moderate and highly tolerant to the bacterial canker, respectively (Sanver *et al.*, 2022).

Regarding the importance of applying resistant cultivars in plant protection, the resistance response of 24 tomato cultivars was evaluated against Cmm. Three disease indices, including time of disease onset, disease severity (DS), and the area under the disease progress curve (AUDPC), were investigated over 30 days.

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Materials and Methods

Pathogenic bacterial strains

This study was carried out in 2019. Four pathogenic strains of *C. michiganensis* subsp. *michiganensis* were prepared from the microbial collection of the Department of Plant Protection of Urmia University. These pathogenic strains were isolated from tomato fields in West Azerbaijan province (Aghazadeh *et al.,* 2017). In a previous study, the used strains showed different pathogenicity power on cv. Early Urbana 111, a sensitive tomato cultivar. Purified strains were cultured on a nutrient agar medium for routine use and stored at 25 °C for two days. For long-term storage, freshly cultured colonies were suspended in the 25% glycerol in nutrient broth medium and stored at -80 °C (Schaad *et al.,* 2001).

Plant material

Twenty-four tomato (*Solanum lycopersicum* L.) cultivars commonly cultivated in Iran were used. Studied cultivars were included 13 varieties (Early Urbana 111, Early Urbana Y, King Stone, Super 22 TO, CalJ N3, 2270, Rio Grenade, Early Urbana, Primo Early, Falat CH, Super Chef, Primax, and Red Stone), and 11 hybrids (Hyb. 6515, Hyb. Superset, Hyb. Firenze, Hyb. Comodoro, Hyb. Bellariva, Hyb. 1585, Hyb. Kishmat, Hyb. Eden, Hyb. 8320, Hyb. Monty marker F1, and Hyb. Ferguson F1).

Inoculation of tomato seedlings

Tomato seeds were surface disinfested by ethanol 70% for two minutes, then sodium hypochlorite 2% for three minutes, and then washed three times with sterile distilled water (SDW). Seeds were sown in culture trays containing an equal mixture of clay and sand. When the seedlings reached 2-3 leaf stage, they were transferred to plastic pots containing a mixture of clay, sand, and compost in equal proportions. Tomato seedlings at the 4-5 leaf stage were injected with a suspension of 1×10^4 CFU ml^{-1} of a combination of four pathogenic bacterial strains using a syringe at the axil where the petiole meets the stem. To preserve moisture, the injection site or wound was covered with Parafilm. The control plants were injected with sterile distilled water. The experiment was repeated twice in a greenhouse with controlled conditions under natural daylight, at 28 °C, and 80% relative humidity (RH). The experiments were repeated twice in the greenhouse (de León *et al.,* 2008).

Evaluation of disease indexes

Three indexes, including the time of disease onset, the disease severity, and the area under the disease progress curve (AUDPC), were used to evaluate the response of cultivars to the disease. Therefore, disease symptoms were recorded daily from the day after inoculation up to 30 days later.

Disease severity was evaluated at the end of the experiments under a 1 to 5 scoring scale: 1) no symptoms (resistant), 2) wilting in 1–25% of each plant (tolerant), 3) wilting in 26–50% of each plant (moderately tolerant), 4) wilting in 51–75% of each plant (susceptible), and 5) wilting in 76–100% of each plant or dead plant (very susceptible) (Klement *et al.,* 1990).

The disease severity for each cultivar was calculated based on the following formula (Valenzuela *et al.,* 2021):

$$DS = \frac{\sum(n \times x_{1-5})}{N \times 5} \times 100$$

Where *n*: is the number of plants in each numerical score, $x_{1\text{-}5}$: is the numerical score, and *N*: is the total number of evaluated plants for each cultivar.

The area under the disease progress curve was calculated via the following formula (Ialacci *et al.,* 2016).

$$AUDPC = \sum_{i=1}^{n_{i-1}} \frac{y_i + y_{i+1}}{2} (t_{i+1} - t_i)$$

Where y_i: the disease severity in *i*th observation, y_{i+1}: the disease severity in $i + 1$ observation, t_i: the recording time in *i*th observation, t_{i+1}: the recording time in $i + 1$ observation, and *n*: the total number of observations.

Statistical analysis of data

Pot experiments were conducted with a completely randomized design (CRD). Four repeats (sample) were considered for each

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treatment (cultivar), and five seedlings in separated pots were assigned as subsamples. Statistical analysis of data was performed by Tukey test ($P \leq 0.01$), using SAS software (version 9.4). The AUDPC index was calculated using R software (version 3.5.2) and the Agricolae package. The rank correlation between disease resistance indexes was evaluated using the Spearman correlation coefficient in SPSS software (version 25). Results are the average data of the two separate experiments conducted in the greenhouse.

Results

The reaction of 24 tomato cultivars was evaluated against the bacterial pathogen *C. michiganensis* subsp. *michiganensis* under greenhouse conditions. Data analysis of variance indicated a significant difference between tomato cultivars in resistance response against tomato bacterial canker (Table 1).

The first symptoms of the disease appeared three to 15 days following inoculation. A data comparison revealed a significant difference at $P \leq 0.01$, and the cultivars were divided into different statistical groups (Table 2). Cv. Super 22 TO showed disease symptoms three days after inoculation, which was the most sensitive cultivar regarding the mentioned feature. Symptoms appear in Super Chef, Falat CH, Red Stone, Hyb. Comodoro and Hyb. Eden four to seven days after inoculation, but no statistically significant difference with Super 22 TO was observed. Symptoms appeared in Rio Grenade later than other cultivars, about 15 days after inoculation. Based on the results of this feature, 11 cultivars, including King Stone, Early Urbanae, Hyb Superset, and Early Urbana Y have no statistically significant difference with Rio Grenade, so they were evaluated as the most resistant cultivars in symptoms appearance (Table 2).

Based on the disease symptoms, a numerical scoring scale of one to five degrees was used to assess the disease severity index (Fig. 1) (Klement *et al.*, 1990). Disease severity of Early Urbana 111 and Hyb. 6515 was 83.33% and showed the highest disease severity in studied cultivars. Cvs. King Stone, Early Urbana Y, Hyb. Firenze and CalJ N3 showed the lowest disease severity (16%); after those, 13 cultivars, including cvs. Rio Grenade, Primo Early, and Hyb. 8320 with a disease severity from 22.16-44.50% were placed. Therefore, based on the disease severity, the mentioned cultivars showed more resistance to the disease than other studied cultivars (Table 2).

By comparing the mean area under the disease progress curve, Rio Grenade had the lowest AUDPC and was identified as the most resistant cultivar based on AUDPC value. Cultivars King Stone, Early Urbana Y, CalJ N3, and Hyb. Firenze were also located in the same statistical group as Rio Grenade, and they were the most resistant cultivars based on this index. The highest AUDPC was observed in Super Chef, so this cultivar showed the most sensitivity to the disease among the studied cultivars (Table 2).

The correlation coefficient between the three disease response indexes showed that time of disease onset had a positive correlation at 1% level with disease severity ($r = 0.54^{**}$) and AUDPC ($r = 0.85^{**}$), as well as AUDPC and disease severity, showed a positive correlation ($r = 0.86^{**}$) at 1% level (Table 3).

Table 1 Variance analysis of pathogenicity indexes of tomato against *Clavibacter michiganensis* subsp. *michiganensis*.

		Mean squares		
Source of variations	Degrees of freedom	Time of disease onset	Disease severity	AUDPC[1]
Tomato cultivars	23	34.86 **	5.71 **	246.1 **
Experimental error	48	1.93	0.31	14.18
Coefficient of variabilities (%)		14.21	22.36	22.94

[1] The area under the disease progress curve.
[2] **Significance level at 1%.

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Table 2 The response of different tomato cultivars against bacterial canker disease by measuring three indices: time of disease onset, disease severity, and the area under the disease progress curve.

No	Cultivar	Time of disease onset (day)	Grade	Disease severity (%)	Grade	AUDPC[1]	Grade
1	Rio grenade	15.33 ± 0.88 [a 2]	1	22.16 [de]	2	3.67 ± 0.44 [g]	1
2	King stone	14.33 ± 0.88 [ab]	2	16.00 [e]	1	4.50 ± 1.00 [g]	2
3	Early Urbana	13.67 ± 0.67 [ab]	3	33.33 [de]	5	7.67 ± 1.33 [fg]	7
4	Hyb. Superset	13.33 ± 1.67 [abc]	4	27.83 [de]	4	9.17 ± 2.89 [efg]	8
5	Early Urbana Y	13.00 ± 0.00 [a-d 3]	5	16.00 [e]	1	5.50 ± 0.00 [g]	3
6	CalJ N3	12.67 ± 0.33 [a-d]	6	16.00 [e]	1	5.83 ± 0.33 [g]	4
7	Primo early	12.33 ± 0.33 [a-e]	7	22.16 [de]	2	7.30 ± 1.36 [fg]	6
8	Hyb. Firenze	12.00 ± 0.00 [a-f]	8	16.00 [e]	1	6.50 ± 0.00 [g]	5
9	Hyb. Monty marker F1	12.00 ± 1.00 [a-f]	8	72.16 [abc]	3	18.80 ± 4.05 [a-f]	14
10	Primax	11.67 ± 0.33 [a-f]	9	33.33 [de]	5	10.67 ± 0.67 [d-g]	10
11	Hyb. 8320	11.33 ± 0.67 [a-g]	10	22.16 [de]	2	9.67 ± 2.68 [d-g]	9
12	Hyb. Ferguson F1	10.67 ± 0.67 [b-h]	11	72.16 [abc]	9	21.50 ± 2.18 [a-d]	16
13	Early Urbana 111	9.00 ± 0.00 [c-i]	12	83.33 [a]	11	24.80 ± 1.86 [abc]	19
14	2270	9.00 ± 1.00 [c-i]	12	27.83 [de]	4	14.17 ± 3.32 [c-g]	11
15	Hyb. 6515	8.67 ± 0.33 [d-i]	13	83.33 [a]	11	25.80 ± 0.33 [abc]	20
16	Hyb. Kishmat	8.67 ± 1.33 [d-j]	13	77.83 [ab]	10	28.30 ± 1.83 [ab]	22
17	Hyb. 1585	8.00 ± 2.00 [e-j]	14	38.83 [de]	6	18.50 ± 2.60 [c-f]	13
18	Hyb. Bellariva	7.67 ± 0.33 [f-j]	15	27.83 [de]	4	15.50 ± 2.57 [c-g]	12
19	Hyb. Eden	7.00 ± 0.00 [g-k]	16	72.16 [abc]	9	29.20 ± 1.45 [ab]	23
20	Hyb. Comodoro	6.33 ± 0.88 [h-k]	17	33.33 [de]	5	21.00 ± 2.52 [a-e]	15
21	Falat CH	5.33 ± 0.67 [ijk]	18	44.50 [cde]	7	24.30 ± 2.80 [abc]	18
22	Red Stone	5.33 ± 0.33 [ijk]	18	44.50 [cde]	7	27.70 ± 2.49 [ab]	21
23	Super Chef	4.33 ± 0.33 [jk]	19	50.00 [bcd]	8	30.50 ± 3.75 [a]	24
24	Super 22 TO	3.00 ± 0.00 [k]	20	33.33 [de]	5	23.3 ± 1.86 [abc]	17

[1] The area under the disease progress curve
[2] In each column, the numbers with dissimilar letters have a significant difference of 1%, based on the Tukey's test.
[3] The hyphen between the letters of the statistical groups was to prevent lengthening letters; a-d means the statistical group of abcd.

Figure 1 Disease severity degrees of tomato bacteria canker, 1) no symptoms (resistant), 2) wilting in 1–25% of plant (tolerant), 3) wilting in 26–50% of plant (moderately tolerant), 4) wilting in 51–75% of plant (susceptible), 5) wilting in 76–100% of plant or dead plant (very susceptible), and C) control.

Table 3 The correlation coefficient between three resistance indexes of tomato cultivars against bacterial canker disease.

	Time of disease onset	Disease severity	AUDPC[1]
Time	1.00		
Disease severity	**0.54	1.00	
AUDPC	**0.85	**0.86	1.00

[1] The area under the disease progress curve.
*Significance level at 1%.

Discussion

This study investigates the reaction of 24 tomato cultivars against tomato bacterial canker disease. A comprehensive knowledge of host and pathogen biology and their interactions is needed to provide accurate disease management strategies (Peritore-Galve *et al.*, 2021). Pathogen-

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free seeds and disease-resistant or -tolerant cultivars are the most effective and safest strategies in this disease management (Sen *et al.,* 2015). Understanding different aspects of host resistance can help identify genes involved in tolerance or resistance commercial cultivars through traditional breeding and transgenic approaches (Peritore-Galve *et al.,* 2021). Many tomato germplasm collections were evaluated to find new resistance sources to bacterial canker (Abebe *et al.,* 2022). However, there are a few cultivars with significant resistance or tolerance to this disease and high-quality fruits (Yang and Francis, 2007; de León *et al.,* 2011; Nandi *et al.,* 2018).

To the best of our knowledge, there is no information about the degree of resistance in the studied cultivars based on three parameters: the time of disease onset, disease severity, and AUDPC. The results of the onset of the disease indicated that the symptoms became evident 3-5 days after inoculation in sensitive cultivars such as cvs. Super 22 TO and Super Chef. In agreement with our study, Tsitsekian et al. (2021) reported that symptoms of the disease appeared on the third day in sensitive cultivars, reaching the highest level following six days and remained constant until the twelfth day after inoculation. Hibberd et al. (1992) reported Heinz 2990 showed more resistance than Morden and Floradade genotypes. The first symptom appearance and disease development in Heinz 2990 was later than the other two genotypes. In addition, IRAT L3 (Sen *et al.,* 2013) and LA2157 (Kabas *et al.,* 2018) genotypes are also reported as the most resistant. It seems that resistance to Cmm in tomato cultivars differed and depended on the sources of resistance, and this resistance can be achieved with molecular methods such as QTL mapping (Wang *et al.,* 2018).

The present study considers the results of three studied indexes together, cvs. Rio Grenade, King Stone, Early Urbana Y, CalJ N3, and Hyb. Firenze were determined as the most resistant cultivars, and cvs. Super Chef, Hyb. Eden, Hyb. Kishmat and Red Stone were identified as higher susceptible cultivars to Cmm; however, differences between these cultivars' rankings in the three studied indexes were observed, for instance, in Hyb. Monty marker F1 first symptom appears after 12 days, but in terms of disease severity and AUDPC indexes, it was the sixth and eleventh sensitive cultivars, respectively. Resistance response to Cmm is multigene (Peritore-Galve *et al.,* 2021; Yokotani *et al.,* 2021). Such differences may be due to gene interactions that environmental conditions can affect (Sen *et al.,* 2015). Indeed, investigation of genetic resistance and understanding of the mechanism of plant response towards Cmm can better clarify the basis of resistance and susceptibility in the evaluated cultivars (Peritore-Galve *et al.,* 2021; Yokotani *et al.,* 2021). Usage of resistance cultivars led to slight wilting of the plant despite the high density of the bacteria (Sen *et al.,* 2015).

The correlation coefficient results between the three disease indices indicated that the AUDPC value is more reliable than the others. Consequently, 12.5% of cultivars are scored as very susceptible, 12.5% as susceptible, 42% as moderately tolerant, and 33% as tolerant. No resistant cultivar was found among the studied cultivars.

Nerveless, Cvs. Rio Grenade, King Stone, Early Urbana Y, CalJ N3, and Hyb. Firenze showed acceptable tolerance to bacterial canker disease and may be recommended in integrated management programs in the future.

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Statement of conflicting interests

The authors state that there is no conflict of interest.

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واکنش مقاومت به باکتری *Clavibacter michiganensis* subsp. *michiganensis* در ارقام مختلف گوجه‌فرنگی موجود در ایران

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چکیده: شانکر باکتریایی گوجه‌فرنگی ناشی از باکتری *Clavibacter michiganensis* subsp. *michiganensis*، یک بیماری مخرب گوجه‌فرنگی در جهان و ایران است که می‌تواند کیفیت محصول را به‌شدت تحت تأثیر قرار دهد. برای کنترل بیماری، یک برنامه مدیریت تلفیقی مبتنی بر استفاده از بذرهای عاری از بیماری و ارقام مقاوم ضروری است. در این پژوهش، واکنش ۲۴ رقم گوجه‌فرنگی، شامل ۱۳ واریته و ۱۱ هیبرید در برابر این بیماری در شرایط گلخانه مورد بررسی قرار گرفت. مایه‌زنی گیاهچه‌های گوجه‌فرنگی در مرحله ۵-۴ برگی با تزریق سوسپانسیون باکتریایی 1×10^{4} واحد تشکیل‌دهنده پرگنه بر میلی‌لیتر (OD_{600}) در محل اتصال دمبرگ به ساقه اصلی انجام شد. واکنش ارقام به بیماری با استفاده از سه شاخص زمان مشاهده اولین علائم بیماری، شدت بیماری (DS) و سطح زیر منحنی پیشرفت بیماری (AUDPC) ارزیابی شد. نتایج نشان داد که AUDPC با زمان مشاهده اولین علائم بیماری (r= ۰/۸۵) و شدت بیماری (r= ۰/۸۶) هم‌بستگی مثبت دارد. براساس یافته‌های این مطالعه، درنظر گرفتن شاخص‌های مختلف در واکنش ارقام گیاهی به بیماری‌ها، اطلاعات دقیق‌تری در مورد مقاومت به بیماری ارائه می‌دهد و شاخص AUDPC به‌دلیل داشتن ضریب هم‌بستگی مثبت بالا با دو شاخص دیگر، قابل اعتمادتر است. در این مطالعه ارقام Rio Grenade، King Stone، Early Urbana Y، CalJ N3 و Hyb. Firenze نسبت به سایر ارقام مورد مطالعه، مقاومت بیش‌تری در برابر این بیماری نشان دادند و استفاده از ارقام ذکر شده در برنامه مدیریت تلفیقی بیماری شانکر باکتریایی گوجه‌فرنگی توصیه می‌شود.

واژگان کلیدی: AUDPC، شانکر باکتریایی، آلودگی آوندی، مقاومت به بیماری

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