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Resistance Evaluation of Some Commercial Strawberry Cultivars to Anthracnose Fruit Rot Caused by *Colletotrichum Nymphaeae* under *in Vivo* and Greenhouse Conditions

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ABSTRACT

Strawberry Anthracnose Fruit Rot (AFR) is one of the main limiting factors for strawberry production industry worldwide including Iran. Due to the restrictions associated with fungicides application across strawberry fields, their adverse effects on environment and the possible fungicides resistance development among fungal strains of the pathogen, the use of resistant cultivars is considered the most effective method for the management of this disease. In this study, reactions of 25 commercial strawberry cultivars were evaluated against the fungus *Colletotrichum nymphaeae*, causing strawberry AFR using fruit, leaf, and crown-based assays. According to the results of this study, the strawberry cultivars showed different reactions to the disease depending on the inoculation of their leaf, fruit, and crown with *C. nymphaeae* PET1 under *in vivo* and greenhouse conditions. However, fruit-based assay was a better indicator of AFR disease susceptibility due to nature of AFR disease caused by *C. nymphaeae*. Overall, 'Blakemore' and 'Kurdistan' cultivars were significantly more resistant compared with others, except 'Aliso', 'Mrak', 'Diamant', 'Yallova', 'New Kurdistan', 'Mac Donance' and 'Ten Beauty', respectively. On the contrary, 'Gaviota' cultivar was significantly the most susceptible than the rest, except 'Camarosa'. The common commercial cultivars grown in Iran including 'Camarosa', 'Paros', 'Pajaro', and 'Queen Eliza' were categorized on the list of susceptible and highly susceptible cultivars in this study. To the best of our knowledge, this is the first study evaluating the reaction of some commercial strawberry cultivars against *C. nymphaeae* causing strawberry AFR.

Keywords: *Fragaria×ananassa* Duch., *In vivo* and greenhouse assays, Resistant cultivars, Strawberry AFR.

INTRODUCTION

Strawberry (*Fragaria×ananassa* Duch.) is a widely grown hybrid species in the family Rosaceae, which is globally appreciated for its fruit. Strawberry is becoming a major product for agricultural economic development of developing countries including Iran, with Kurdistan being the leading strawberry-producing province where strawberry is produced predominantly under open-field conditions (Tehranifar and Sarsaefi, 2002). Many biotic and abiotic limiting factors are responsible for the reduction of strawberry production worldwide. For instance, more than 100 fungal species can invade strawberry plant, one-third of which make serious diseases on the different parts of the plant (Martin and

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Spiegel, 1998). Anthracnose is an important disease of strawberry caused by *Colletotrichum* spp., which appears on foliage, runners, crowns and fruits (Howard *et al*., 1992). Principally, three fungal pathogens of *C. acutatum* sensu lato, *C. gloeosporioides* sensu lato, and *C. fragariae* are known to be responsible for causing anthracnose disease on strawberry (Freeman and Katan, 1997). Crown rot syndrome is typical of *C. gloeosporioides* sensu lato and *C. fragariae* infections, whereas fruit rot is mainly caused by *C. acutatum* sensu lato leading to a considerable yield loss (Howard *et al*., 1992). To data, a large number of species described are accepted in what is now known as the *C. acutatum* complex, of which six species *viz.*, *C. acutatum sensu stricto*, *C. fioriniae*, *C. godetiae*, *C. nymphaeae*, *C. salicis* and *C. simmondsii* have been revealed to be associated with strawberry anthracnose worldwide (Damm *et al*., 2012). *Colletotrichum nymphaeae* is the most widespread pathogen causing strawberry anthracnose worldwide including Iran (Baroncelli *et al*., 2015; Karimi *et al*., 2017; Wang *et al*., 2019). No chemical disease control methods are available to effectively control the strawberry anthracnose and their use is limited to preventive treatments applied before fruit production (Denoyes-Rothan *et al*., 1999). Moreover, serious toxic effects of environmental chemical fungicides and the development of resistance among fungal strains are of main concerns associated with chemical fungicides application. Hence, the use of resistant cultivars seems to be the most efficient and practical control strategy for suppressing anthracnose development. Given the recent outbreaks of anthracnose disease across strawberry fields in Kurdistan province of Iran (Karimi *et al*., 2017) and considering the need to adopt appropriate strategies in dealing with this challenge, our aim in this study was to assess the resistance of some commercial strawberry cultivars to the anthracnose fruit rot caused by *C. nymphaeae* under *in vivo* and greenhouse conditions.

MATERIALS AND METHODS

Field Surveys and Sampling

An extensive survey was performed in strawberry fields in the Kurdistan province of Iran, particularly in the counties with the main strawberry production including Sanandaj, Mariwan, Kamyaran and Sarvabad. Typical symptoms of the disease was monitored and samples were collected from symptomatic plant parts showing leaf spot, stem necrosis, and/or fruit rot and transferred to the laboratory in single sterile boxes.

Pathogen Isolation and Identification

Edges of lesions having infected parts disinfected in sodium hypochlorite (1%) for 30 seconds, followed by 70% ethanol for 60 seconds, and rinsed in sterile distilled water three times for 10 seconds. Sterilized segments were left to dry on sterile paper under a laminar flow hood and cultured on PDA (Merck Company, Germany). Plates were incubated at 25°C for 7 days in the dark, then, single spore cultures were prepared. The cultures were preserved on potato carrot agar slants (PCA; extract from 20 g potatoes and 20 g carrots, 15 g agar, 1,000 mL distilled water) at 4°C for further use. For morphological identification, macroscopic (colony color, shape and growth) and microscopic (conidial size and shape, presence of seta) features of isolates were studied on PDA after 7 days of incubation at 25°C in the dark.

Pathogenicity Test

Based on fulfill Koch's postulates, the pathogenicity of *Colletotrichum*-like isolates was evaluated on detached untreated fruits of *Fragaria* × *ananassa* cv. Camarosa. In brief, the fruits were surface-disinfected by dipping in 1% sodium hypochlorite,

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followed by 70% ethanol for 30 s, and rinsed three times in sterile distilled water. They were then left to dry up on sterile paper under a laminar flow hood. Three hundred µL of conidial suspension (1×10^6 conidia mL^{-1}) of each isolate containing Tween 80 (0.1%; v/v) was placed on each fruit. Each treatment included five fruits and sterile distilled water containing 0.1% Tween 80 was used as the untreated control. After 7 day, disease incidence and severity were calculated based on the disease index as described by Karimi *et al*. (2017). For calculation of disease severity, total surface area of each strawberry fruit, imagined as having a conical shape, was approximately measured and finally the rate of disease severity was calculated using the following formula: t/T × 100; where (t) and (T) are the infected and total areas respectively of each strawberry fruit.

Cultivars

All strawberry cultivars used in this study were obtained from the strawberry collection of Kurdistan Agricultural and Natural Resources Research and Education Center, Sanandaj, Iran (Table 1).

Detached Leaf Assay

Detached untreated leaves of selected commercial strawberry cultivars were initially surface-sterilized by dipping in 1% sodium hypochlorite for 30 seconds and ethanol 70%, followed by rinsing three times in sterile distilled water. The leaves were allowed to dry up on sterile paper under a laminar hood before inoculation. Inoculation was performed as described by Karimi *et al*. (2019). Based on the leaf size, four to 12 wounds were made on the adaxial surface of each leaf using a sterile sharp needle. For inoculation, a plug of 5 mm of seven-day-old fungal culture of *C. nymphaeae* PET1 was put on the wounds of each leaf. After inoculation, leaves were kept in plastic containers with moistened filter papers at 23°C to keep high relative humidity (> 95%). Five detached leaves were inoculated for each fungal strain and pathogen-free PDA plugs were served as control (Figure. 1). After 8 days, affected area on leaf surface was measured using imageJ v. 1.51i software (https://imagej.nih. gov/ij/index.html) for the calculation of disease severity.

Detached Fruit Assay

Detached untreated fruits of each cultivar were inoculated with the conidial suspension of *C. nymphaeae* PET1 at concentration of 1×10^6 conidia mL^{-1} containing Tween 80 (0.1%; v/v) as described in pathogenicity test. Six fruits were inoculated for each cultivar. After 7 days, disease incidence and severity were calculated based on the above-mentioned index recommended.

Greenhouse Assay

In the greenhouse, crowns of one-year-old strawberry plants were injected by the *C. nymphaeae* PET1 to produce crown rot symptoms in different cultivars. One mL conidial suspension of *C. nymphaeae* PET1 containing Tween 80 (0.1%; v/v) adjusted to 1×10^6 conidia mL^{-1} was injected into crown tissue of each cultivar using a sterile syringe. Four plants for each cultivar were inoculated. The plants injected with sterile distilled water were used as negative control. After inoculation, plants were initially kept under conditions of high humidity (> 92%) with a 16 hour light/8 hour darkness at 26°C for 48 hours. Afterwards, the plants were transferred to a greenhouse with a 12-hour photoperiod at 26°C for four months and the appearance of crown rot symptoms was assessed based on the following disease index recommended by Smith and Black (1987): 0= Healthy plant with no visible lesions; 1= Plant with petiole lesions< 3 mm long; 2= Plant with petiole lesions 3-10 mm long; 3= Plant with petiole lesions> 10-20 mm long, usually gridding the petiole; 4= Plant with petiole lesions> 20 mm

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Table 1. Strawberry cultivars used in this study and their origins.

Name	Pedigree	country
Aliso	A self of Cal.52.16-12	USA
Aromas	Cal 87.112-6×Cal 88.270-1	USA
Blakemore	Missionary×Howard 17	USA
Camarosa	Douglas×Cal 85.218-605	USA
Chandler	Douglas×Cal 72-361-105	USA
Dachnitsa	Venta×Tenira	Belarus
Diamant	-	USA
Fresno	Lassen×Cal 83.25-2	USA
Gaviota	Cal 87.112-6×Cal 88.270-1	USA
Karcynberg	Venta×Tenira	Belarus
Kurdistan	-	USA
Mac Donance	-	USA
Missionary	A chance seedling in USA,1900	USA
Mrak	CN27(Ca75.34-105)	USA
N. Selva	-	USA
New Kurdistan	-	USA
Pajaro	Cal 63.7-101×Sequoia	USA
Paros	Marmolada×Irvine	Italy
Queen Eliza	Missionary×USB 35	Italy
Selva	Cal 70.3-117×Cal 71.98-605	USA
Sequia	Cal 52.16-15×Cal 51s 1-1	USA
Ten Beauty	Howard 17×Missionary	USA
Ventana	Senga Sengana×Festival naja	Lithuania
Ventana1	-	Lithuania
Yalova	Arnavutkoy×Aliso	Turkey

Figure 1. Symptoms of anthracnose fruit rot (A), sunken lesion on stolon (B), flower blight (C) and crown rot (D) observed on naturally infected plants of strawberry caused by *Colletotrichum nymphaeae* collected from strawberry fields of Kurdistan province in Iran. Inoculation of leaves (E and H), fruits (F and I) and crowns (G and J) of strawberry cultivars with *C. nymphaeae* PET1 in this study and their reactions.

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long to entire petiole necrotic; 5= Plant whose youngest leaf was wilted; and 6= Dead plant with necrotic crown.

Statistical Analyses

Data were statistically analyzed as a Completely Randomized Design (CRD) by standard Analysis Of Variance (ANOVA).

The means were compared with critical difference and presented as mean±Standard Error (SE). All analyses were carried out using SAS software (SAS institute, Inc., 2003).

RESULTS AND DISCUSSION

During the survey of strawberry fields, the symptoms associated with anthracnose disease were mainly observed as AFR, while other syndromes including flower blight, sunken fusiform lesions on runners, V-shaped necrotic lesions around the principal vein, and stunted and semi-dried plants were also visible [Figure 1 (A-D)]. These observations were in line with those reported by Karimi *et al*. (2017). Of symptomatic plant tissues, totally eight *Colletotrichum*-like fungal isolates were obtained from fruit (six isolates) and the rest from crown (one isolate) and leaf (one isolate). Based on macroscopic (white to grey colony with the average growth rate of 60 mm after 7 days) and microscopic (sexual form, sclerotia, chlamydospore and seta were absent; conidia were 15.25–17.75×3.9–5 in size µm, unicellular, hyaline, smooth-walled, and cylindrical to fusiform, mostly with both ends acute or with one end acute and one end round), morphological characteristics of all fungal isolates on PDA, their identity were determined as *C. nymphaeae* (Damm *et al*., 2012; Karimi *et al*., 2017). This result further corroborated the *C. nymphaeae* as the sole casual agent of strawberry anthracnose in Iran, as previously reported by Karimi *et al*. (2017). In pathogenicity test, all *C. nymphaeae* isolates were able to induce AFR symptoms on the fruits of cv. Camarosa resembling those observed in the field, although no significant disease incidence or severity differences were detected between isolates (data not shown) [Figure 1 (E-J)]. This high level of the homogeneity in pathogenicity among fungal isolates in this study further confirmed the possibility of the spread of pathogen populations from a single or few sources of origin as noted by Karimi *et al*. (2017). However, based on optical comparisons, *C. nymphaeae* strain PET1 was selected for further use in our examinations. *Colletotrichum nymphaeae* is also the predominant causal agent of strawberry anthracnose in other courtiers including USA and UK (Baroncelli *et al*., 2015; Wang *et al*., 2019). Furthermore, it appears that broadleaf weeds can act as the reservoir of the pathogen inoculums (Karimi *et al*., 2019). These evidences reveal the need for further understanding of the disease epidemiology to improve the management of the disease.

In this study, distinct levels of resistance to AFR were revealed in anthracnose trials of detached leaf, fruit, and crown-based assays. Fruit, leaf, and crown infections were confirmed by re-isolation of the pathogen in all assays. In detached leaf assay, ‘Missionary’, ‘New Kurdistan’, ‘Ventana1’, ‘Ventana’, and ‘Blakemore’ cultivars significantly (df= 24, F= 14.66, P≤ 0.01) exhibited the most resistance to the disease compared with other cultivars Except ‘Ten Beatuy’, ‘N. Selva’, ‘Chandler’, ‘Aliso’, ‘Fresno’, ‘Mac Donance’, ‘Gaviota’, ‘Dachnitsa’, ‘Aromas’ and ‘Mrak’ cultivars, respectively (Table 2). The most susceptible cultivar was ‘Camarosa’, followed by ‘Selva’, ‘Paros’ ‘Diamant’, ‘Karcynberg’, ‘Kurdistan’, ‘Pajaro’, ‘Queen Eliza’, ‘Sequia’ and ‘Yallova’, respectively (Table 2). In detached fruit assay, AFR appeared on all fruits belonging to different cultivars in this assay. However, disease severity was significantly (df= 24, F= 16.85, P≤ 0.01) different between cultivars. ‘Blackmore’ and ‘Kurdistan’ were

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Table 2. Reaction of some commercial strawberry cultivars to the fungus *Colletotrichum nymphaeae* causing strawberry anthracnose fruit rot based on fruit, leaf and crown assays. [a]

Cultivars	DFA		DLA		CIA	
	DS	DI (%)	DS	DI (%)	DS	DI (%)
Aliso	$2.55^{ij} \pm 0.5$	100	$63.78^{fg} \pm 33.05$	100	1.25±2	25
Aromas	$4.01^{fgh} \pm 0.9$	100	$149.04^{defg} \pm 40.79$	100	2.5±2	25
Blakemore	$1.87^{j} \pm 0.4$	100	$36.46^{g} \pm 26.34$	100	1.25±2	25
Camarosa	$8.97^{ab} \pm 0.7$	100	$1226.34^{a} \pm 254.99$	100	1.25±2	25
Chandler	$5.87^{cde} \pm 0.3$	100	$56.7^{fg} \pm 18.67$	100	1.25±2	25
Classica (Dachnitsa)	$6.6^{c} \pm 0.4$	100	$97.46^{defg} \pm 30.95$	60	0.0	0
Diamant	$2.83^{hij} \pm 0.2$	100	$1008.64^{a} \pm 165.00$	100	1.25±2	25
Fresno	$6.07^{cd} \pm 0.5$	100	$64.6^{fg} \pm 14.75$	100	1.5±3	25
Gaviota	$9.67^{a} \pm 0.4$	100	$92.88^{defg} \pm 25.34$	100	1.5±3	25
Karcynberg	$4.32^{fg} \pm 0.3$	100	$1001.44^{a} \pm 182.07$	100	0.0	0
Kurdistan	$2.18^{j} \pm 0.4$	100	$635.24^{b} \pm 390.87$	100	1±2	25
Mac Donance	$3.08^{ghij} \pm 0.4$	100	$81.86^{efg} \pm 42.14$	100	0.0	0
Missionary	$4.87^{def} \pm 0.3$	100	$8.28^{g} \pm 3.8$	80	0.0	0
Mrak	$2.6^{ij} \pm 0.4$	100	$288.02^{cdefg} \pm 54.86$	100	1.25±2	25
N. Selva	$4.38^{fg} \pm 1$	100	$53.62^{fg} \pm 28.37$	80	0.0	0
New Kurdistan	$3.07^{ghij} \pm 0.5$	100	$15.68^{g} \pm 4.08$	100	0.0	0
Pajaro	$5.85^{cde} \pm 0.4$	100	$504.96^{bc} \pm 82.38$	100	0.0	0
Paros	$8.05^{b} \pm 0.2$	100	$1047.98^{a} \pm 202.44$	100	0.25±0.5	25
Queen Eliza	$4.58^{ef} \pm 0.7$	100	$384.92^{bcd} \pm 56.43$	100	1.5±3	25
Selva	$3.68^{fghi} \pm 0.8$	100	$1119.1^{a} \pm 221.25$	100	0.0	0
Sequia	$4.3^{fg} \pm 0.2$	100	$369.32^{bcde} \pm 131.04$	100	1.25±2	25
Ten Beauty	$3.13^{ghij} \pm 0.4$	100	$41.5^{fg} \pm 20.83$	100	0.25±0.5	25
Ventana	$3.87^{fghi} \pm 0.5$	100	$24.32^{g} \pm 10.63$	100	0.0	0
Ventana1	$3.68^{fghi} \pm 0.4$	100	$21.28^{g} \pm 8091$	100	1.5±3	25
Yalova	$2.85^{hij} \pm 0.6$	100	$340.86^{bcdef} \pm 47.34$	100	0.0	0

[a] DFA= Detached Fruit Assay; DLA: Detached Leaf Assay; CIA: Crown Infection Assay; DS= Disease Severity; DI= Disease Incidence. (a-j) Means sharing common letters are not significantly different at $P \leq 0.01$.

significantly the most resistant cultivar to AFR compared with other cultivars besides 'Aliso', 'Mrak', 'Diamant', 'Yallova', 'New Kurdistan', 'Mac Donance' and 'Ten Beuty', respectively (Table 2). On the contrary, 'Gaviota' was highly susceptible to the disease significantly compared with the other cultivars besides 'Camarosa' (Table 2). In greenhouse assay, the occurrence of anthracnose symptoms in strawberry plants inoculated with the *C. nymphaeae* PET1 was considerably low after four months. Statistical comparisons revealed no significant difference between strawberry cultivars in terms of disease incidence and severity. Only one replication of each treatment (cultivar) showed anthracnose crown rot in greenhouse (Table 2). In fact, the wilting and sudden death of the strawberry plant caused by anthracnose crown rot develops and progresses slowly and could start after 30 days of inoculation (Arroyo *et al*., 2009). In this study, wilting and sudden death syndromes were recorded, but different degrees of mild crown rot (brown spots) were ignored. However, latent infection in strawberry farms have been reported earlier (Debode *et al*., 2015). Moreover, a variation in aggressiveness of different isolates of *C. acutatum* as a complex species, and even in *C. nymphaeae* isolates, has been previously proven (Baroncelli *et al*., 2015; Karimi *et al*., 2017).

In all replicates of each treatment, despite the high occurrence of the disease in leaf and fruit-based assays, no linear correlation

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was detected between these assays in terms of disease severity (Correlation coefficient= 0.1445; Determination coefficient R^2= 0.0209). Overall, different and non-identical reactions of cultivars to the disease were detected in detached leaf, fruit, and crown-based assays. For instance, 'Blackmore' was found as a resistant cultivar in detached fruit assay, whereas in detached leaf, it was susceptible. However, the indigenous cultivars of 'Kurdistan' and 'New Kurdistan' were categorized as relatively resistant in both assays. On the contrary, most cultivars showed no crown infection in greenhouse trial (Table 2). These results were in agreement with other studies where the susceptibility to AFR of different organs of a cultivar including fruit, leaf, and root was different and no relationship was detected between the susceptibility of different cultivars and different organs of one cultivar (Delp and Milholland, 1980; Smith and Spires, 1982; Smith and Black, 1990; Chandler *et al*., 1997). Furthermore, the presence of the pathogen may cause no symptoms on the foliage and a latent infection occurs. Overall, similar to Shuman (2001), our results show that the detached fruits assay is the better indicator of AFR disease susceptibility, although other methods could be useful for better understanding of the disease epidemiology. In this study, most commercial cultivars including 'Paros', 'Queen Eliza', 'N. Selva' and 'Camarosa', which are widely grown in strawberry fields of Iran, were categorized as susceptible. Therefore, it seems that the deployment of relatively resistant cultivars such as 'Blackmore', 'Aliso', 'Mark', 'Diamant' and 'Yallova' could be useful in prevention of disease development in the areas where the disease is epidemic. Furthermore, the cultivars with relative resistance can be crossed with commercial cultivars in breeding programs to improve and develop resistant cultivars. To the best of our knowledge, this study is the first attempt to evaluate the reaction of some commercial strawberry cultivars against *C. nymphaeae* causing strawberry AFR.

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ارزیابی مقاومت ارقام تجاری توت فرنگی به بیماری پوسیدگی آنتراکنوزی میوه با عامل *Colletotrichum nymphaeae* تحت شرایط *in vivo* و گلخانه

س، بهرامی کمانگر، ک، کریمی، ف. کرمی،ک، شریفی واش فام، و ک. بهمنی

چکیده

پوسیدگی آنتراکنوزی میوه توت فرنگی (Anthracnose fruit rot) یکی از مهمترین عوامل محدودکننده صنعت تولید توت فرنگی در سراسر جهان از جمله ایران است. با توجه به محدویت‌های مرتبط با کاربرد قارچ‌کش‌ها در مزارع توت فرنگی، تأثیرات منفی آنها بر محیط زیست و نیز احتمال بروز مقاومت در سویه های قارچی بیمارگر به قارچ‌کش‌ها، استفاده از ارقام مقاوم موثرترین روش برای مدیریت این بیماری به حساب می‌آید. در این تحقیق، واکنش ۲۵ رقم توت فرنگی تجاری در مقابل بیمارگر *Colletotrichum nymphaeae* عامل پوسیدگی آنتراکنوزی میوه توت فرنگی با استفاده از روش‌های ارزیابی میوه، برگ و طوقه بررسی شد. براساس نتایج به دست آمده، بافت‌های برگ، میوه و طوقه ارقام توت فرنگی تلقیح شده با عامل بیماری *C. nymphaeae* PET1 واکنش‌های متفاوتی

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را تحت شرایط *in vivo* و گلخانه نسبت به بیماری نشان دادند. با اینحال به دلیل ماهیت بیماری AFR، ارزیابی مبتنی بر میوه شاخص بهتری از حساسیت به بیماری AFR ناشی از *C. nymphaeae* بود. بر همین اساس ارقام 'Blakemore' و 'Kurdistan' به ترتیب در قیاس با سایر ارقام بجز ارقام 'Aliso'، 'Mrak'، 'Diamant'، 'Yallova'، 'New Kurdistan'، 'Mac Donance' و 'Ten Beauty' به طور معنی‌داری بیشترین مقاومت را از خود نشان دادند. درمقابل، رقم 'Gaviota' به طور معنی‌داری بیشترین حساسیت را نسبت به بقیه ارقام بجز 'Camarosa' از خود نشان داد. اغلب ارقام تجاری رایج در ایران از جمله 'Camarosa'، 'Paros'، 'Pajaro' و 'Queen Eliza' که دراین تحقیق مورد ارزیابی قرارگرفتند، جزو ارقام بسیار حساس و حساس طبقه بندی شدند. براساس اطلاعات موجود، این اولین مطالعه در زمینه ارزیابی واکنش ارقام تجاری توت فرنگی در برابر قارچ *C. nymphaeae* عامل AFR می‌باشد.

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