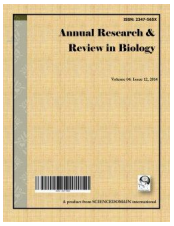

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Neofusiccocum parvum as Uncommon Fungal Species (or Emerging Pathogen) on Strawberry Plants in Morocco

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

During a survey on fungi associated with decline symptoms on strawberry plant of Venicia variety, one species belonging to the Botryosphaeriaceae family was isolated. Based on morphological and cultural characteristics, this species identified as *Neofusicoccum parvum* was reported for the first time in Morocco. To verify the pathogenicity of the fungus, detached leaves of three strawberry varieties were inoculated artificially by depositing over their intact surface mycelia plug or conidial suspension from *N. parvum*. Severity index was greater on festival leaves reaching 88% compared to 77.73% on Sabrina. In the third treatment, Guariguette showed a low susceptibility with a severity index in order of 25.07%. Conidia concentration on the leaf surface of the Festival and Sabrina strawberry leaves was respectively 1.62 10^5 and 1.2 10^5 conidia cm^{-2}. Otherwise, in the second treatment, it has been reduced to less than 1.4110^5 1.16 and 10^5 conidia.cm^{-2} on leaves of Festival and Sabrina respectively. After inoculation, the fungus was re-isolated from the lesions to verify Koch's postulates.

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1. INTRODUCTION

Strawberry (*Fragaria* x *ananassa*) is an important horticultural crop produced both conventionally and organically in open fields, greenhouses, and in plastic covered tunnels [1,2]. In Morocco, the major production districts are located in the main irrigated perimeter of Gharb and Loukkos [3]. The strawberry cultivation offers important opportunities for exporting and generating jobs. By its average of 3500 Ha [3], it contributes for assuring 3000000 and 1500000 working days as seasonal employments at agricultural exploitations or conditioning stations [3]. Moreover, in recent years, exports of fresh and frozen strawberries have known an impressive growth with 16256 tons and 51974 tons respectively in 2014/2015 season [3]. This evolution is due to Moroccan varieties of microclimates, protected agriculture installations, to strong export demand (mainly to Union European), growing domestic consumption, and the propagation of the different strawberry varieties which all have peaks on different moments, guaranteeing a constant supply throughout the whole season [2]. Strawberries, however, are susceptible to wide range of phytopathogenic fungi causing considerable economic losses in crop areas all over the world. A major disease of strawberry is grey mold, caused by *Botrytis cinerea* [4,5]. However, other fungal pathogens affect strawberry plants [6,7,8,9]. Among those, *Podosphaera aphanis* [10,11], *Colletotrichum acutatum* [12], *Phytophthora cactorum* [13,14,15], *Rhizoctonia* spp. [16], *Phytophthora fragariae* [17], *Macrophomina phaseolina* [18], *Verticillium dahliae* [19,20] and *Verticillium albo-atrum* [21]. Furthermore, recently, a new fungal species was isolated from strawberry fruit. It was identified as *Neofusicoccum parvum* which causes postharvest rot and mummification of strawberries [22]. In Morocco, this species was recovered from leaves of collapsed strawberry plants of the Venicia variety collected in Dlalha village [23]. Therefore, the knowledge of the pathogenic capacity of this uncommon fungal species can lead to best management of control measures and correct evaluation of its development risks on strawberry plants.

The present study was initiated to evaluate the pathogenicity of *Neofusicoccum parvum* isolate via the Koch's postulate verification on leaves of strawberry varieties.

2. MATERIALS AND METHODS

2.1 Pathogen Isolation

Diseased tissues from leaves were collected and small pieces of necrotic tissues were surface disinfected and placed onto Petri dishes containing three sterilized filter paper humidified by sterilized distilled water. After 2 to 3 days, the pieces were transferred onto Potato Sucrose Agar (200 g potato, 15 g sucrose, 20 g Agar-Agar, and 1000 mL distilled water) [24]. Plates were incubated at 25°C in the dark and checked regularly. Hyphae growing out from the tissue pieces were subcultured onto PSA and incubated in the darkness at 28°C. Pure cultures were obtained by hyphal tips from the margin of the colonies, which were subcultured on fresh PSA and maintained at 28°C. Cultural characteristics of the isolates including colony color, shape were evaluated on PSA, after 7 days and 20 days of incubation at 28°C in darkness.

2.2 Pathogenicity Test on the Strawberry Leaves

Healthy leaves of three strawberry varieties, Festival, Sabrina and Gariguette were inoculated using three techniques. The surface of leaves was disinfected with 5% sodium hypochlorite, washed with sterile distilled water and dried on a sterile filter paper.

Technique 1: Nine leaves were inoculated with 5 mm of the fungal mycelial plugs and placed in the middle of the intact leaves. Nine leaves were inoculated with 5 mm non-colonized PSA agar plugs were used as controls. The inoculated leaves were further incubated for 7 days at room temperature (25°C).

Technique 2: Nine other leaves were inoculated with the conidial suspension adjusted to a final concentration of 10^5 conidia.mL^{-1} with sterile distilled water containing 0.05% Tween 20 and 5% gelatin. Nine detached leaves with a healthy appearance were simultaneously sprayed with sterilized distilled water without conidia to serve as control. Every brunch of three leaves was placed in 120-mm Petri dish containing slide and humidified with sterile distilled water.

Technique 3: The other nine ones were placed in sterile Petri dishes containing sterile distilled water. A 10 µl drop of spore suspension adjusted

to a final concentration of 10^6 conidia.mL^{-1} with sterile distilled water containing 0.05% Tween 20 and 5% gelatin was deposited on the leave's surface. Nine leaves receiving a 10 µl drop of distilled water amended with 0.05% Tween 20 and 5% gelatin served as control. Petri dishes were further incubated at room temperature (25°C) in the darkness for 7 days.

The diseased leaf area was scored after 7 days of inoculation using the scale of Stover modified by Gauhl et al. [25].

0= No symptoms;
1 = -0.5% of the limbus with symptoms;
2 = 0.6 to 5% of the limbus with symptoms;
3 = 6 to 15% of the limbus with symptoms;
4 = 16 to 30% of the limbus with symptoms;
5 = 31 to 50% of the limbus with symptoms;
6 = 51 to 80% of the limbus with symptoms;
7 = 81 to 100% of the limbus with symptoms.

The severity index (SI) of disease was calculated using the formula:

$$SI = (\Sigma nb/(N - 1) \times T) \times 100$$

n= Number of leaves for each degree of the scale.
b= Degree of the scale.
N= Number of the degrees used in the scale.
T= Total number of the scored leaves.

The conidia production (Conidia.cm^{-2}) of *Neofusicoccum parvum* on the inoculated strawberry leaves was estimated according to the technique of Hill and Nelson [26]. Ten days after inoculation, the leaves which had shown lesions were cut into pieces of 1 cm^2 and placed in 90 mm Petri dishes on three sterile filter paper discs humidified with sterile distilled water. The dishes were incubated for 10 days.

Under continuous fluorescent lighting, each fragment was placed in a test tube containing 1 mL of sterile distilled water and agitated by a vortex mixer for 2 min. The conidia of the pathogen were counted using a Malassez slide under an optical microscope at magnification × 100 with 10 counting of each sample.

3. RESULTS

3.1 Morphological and Cultural Characterization

After 7 days, inoculated strawberry leaves showed necrotic lesions originating from the inoculation point. No lesions developed in the control leaves. Re-isolations from necrotic tissues were successful and the isolate was morphologically identical to that used for inoculations, fulfilling Koch's postulates.

Conidial morphology (shape, cell membrane, color, and presence of septate) from pycnidia was recorded using a binocular microscope at X40 magnification. Colonies on PSA formed abundant aerial mycelium which was initially white but turned dark-gray after 5-6 days at 25°C (Fig. 1C). The reverse side was almost black in older cultures. Three weeks later, external, superficial pycnidia appeared which contained conidiogenous cells that were hyaline with developing conidia (Fig. 1B). Conidia were hyaline when immature, thin-walled, non-septate, smooth, and fusiform to ellipsoidal with a truncate base (Fig. 1A). Conidia frequently become olivaceous or light brown and develop 1 or 2 septa with a darker middle cell and measuring 12.65 × 4.98 µm (Fig. 1A). The obtained conidial dimension in this study and the presence of biseptate conidia showed slight similarities with those previously reported [27,28,29,30]. According to Philips et al. [31], the presence of biseptated conidia was a morphologically distinctive characteristic of *N. parvum.*

Post-inoculation symptoms on strawberry leaves were characterized by the development of a dark brown necrosis that eventually turn into rotting lesions and yellowing of adjacent tissues (Fig. 2A). Superficially, white to grey mycelia grew after 7 days. The mycelial filaments aggregate intensively thereafter and give rise to pycnidia which appear on the surface of the injured areas after 20 to 25 days (Fig. 2C).

The artificial inoculation with *Neofusicoccum parvum* reproduced well-developed dark symptoms on the leaves of both Festival and Sabrina varieties which showed a higher susceptibility in comparison with Gariguette. Both conidia and mycelia inoculation methods proved to be equally pathogenic on strawberry leaves when inoculated with *N. parvum*.

The extent of the obtained necrotic discoloration was considerably larger after the inoculation with a mycelium plug than inoculation with conidial suspension (Table 1).

The same result was reported by Chen et al. [32]. Their study revealed that inoculation of English walnut branches and hulls with a

mycelium plug of Botryosphaeriaceae and Diaporthe spp. resulted in much more severe disease than inoculation with a suspension of conidia.

The estimated disease severity on leaves of Festival and Sabrina strawberry leaves was respectively 88.07 and 77.73 % in the first treatment. As the same, in the second bioassay, festival strawberry leaves were more affected and the disease severity reached respectively 81. 06% and 67.2% (Table 1).

The conidia production on leaf surface was low. Their concentration on strawberry leaves was significantly identical attaining 1.62 10^5 and 1.2 10^5 respectively for Festival and Sabrina strawberry leaves but differs from those collected on Guariguette (Table 2).

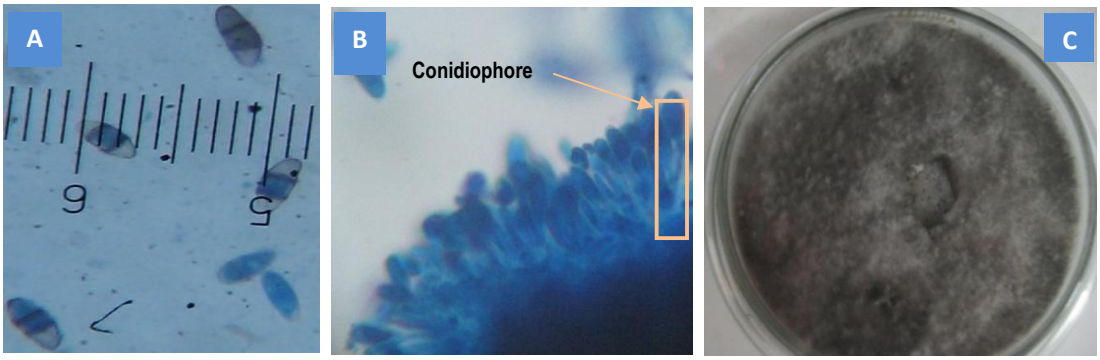

Fig. 1. A: Conidia, B: Conidiophores, C: Culture appearance of *N. parvum* on PSA medium

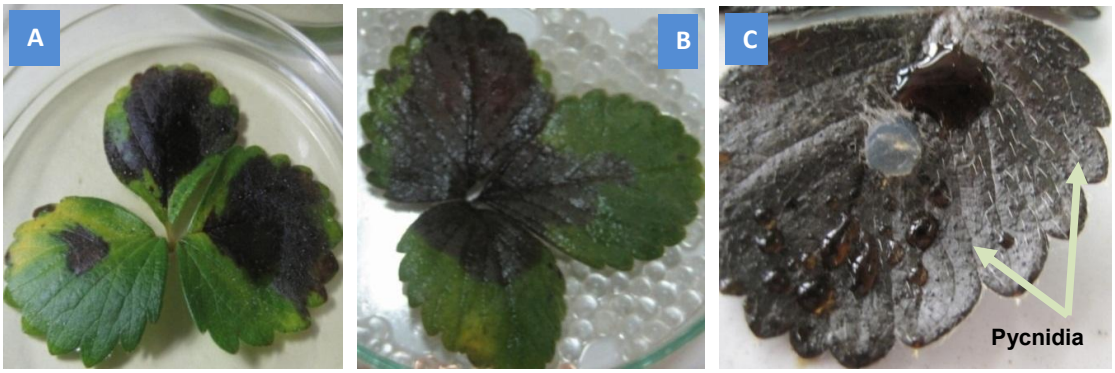

Fig. 2. Developed lesions on strawberry leaves after artificial inoculation by *Neofusicoccum parvum*. Inoculated leaves with a drop of spore suspension (A); inoculated leaves with spore suspension (B); inoculated leaves with mycelia disc (C)

Table 1. Severity diseases on the leaf surface of the inoculated and non inoculated Festival, Sabrina and Gariguette strawberry leaves

	Inoculated strawberry leaves (Festival)	Inoculated strawberry leaves (Sabrina)	Inoculated strawberry leaves (Gariguette)	Control
Treatment 1	88.00 a	77.73a	-	0
Treatment 2	81.6ab	67.2b	-	0
Treatment 3	74.07b	68.33b	25.74	0

The results of the same column followed by different letters differ significantly at 5%.
Treatment 1. Inoculation of the strawberry leaves with mycelia disks.
Treatment 2. Inoculation of the strawberry leaves with conidia suspension.
Treatment 3. Inoculation of the strawberry leaves with drop spore suspension

Table 2. Conidia concentration on the leaf surface of the inoculated and non inoculated Festival, Sabrina and Gariguette strawberry leaves

Treatments	Inoculated strawberry leaves (Festival)	Inoculated strawberry leaves (Sabrina)	Inoculated strawberry leaves (Gariguette)	Control
Treatment 1	1.62a	1.2a	-	0
Treatment 2	1.41a	1.16a	-	0
Treatment 3	1.27a	1.13a	1,01	0

The results of the same column followed by different letters differ significantly at 5%.
Treatment 1. Inoculation of the strawberry leaves with mycelia disks.
Treatment 2. Inoculation of the strawberry leaves with conidia suspension.
Treatment 3. Inoculation of the strawberry leaves with drop spore suspension

4. DISCUSSION AND CONCLUSION

Neofusicoccum parvum on strawberry plant was first reported by Lopes et al. [22]. These authors observed a new fruit rot that was verified in 7% of stored fruits during a survey of strawberry diseases at the postharvest stage. In California, *N. parvum* has been reported from almond [33], avocado [34,35], citrus (*Citrus* sp.) [36], English walnut [32,33], grapevine [37,38], blueberry in Chile [39,40] and *Eucalyptus globules* in North Spain [41]. *N. parvum* was also reported as pathogen on pomegranate in Greece [42] and in the United states [43]. In Italy, this species has a significant importance due to its aggressiveness causing shoot blight and plant decay on pomegranate [44].

Indeed, the taxonomic study on phylogenetic lineages in the Botryosphaeriales accepted *Neofusicoccum* as genus of the family Botryosphaeriaceae [45]. *Botryosphaeriaceae* spp. known to occur worldwide and have been described as plant pathogens, saprophytes, or endophytes of both cultivated and native plants [46,47]. Some members of the Botryosphaeriaceae are latent pathogens. Following physical damage or stress to a host, they often become aggressive and kill large parts of the host [48].

A numerous species of *Neofusicoccum* had been reported as plant pathogens. For instance, *Neofusicoccum vitifusiforme* causes blueberry blight [49], *N. luteum* and *N. parvum* associated with declining grapevines in Portugal in 2002 [28] and *N. mangiferae* causes grapevine dieback in Henan and Anhui Provinces in China [50]. Additionally, Úrbez-Torres et al. [51] also showed the association between *N. parvum* and dark wood streaking symptoms from which the fungus was commonly isolated in California and in seven other states. Among members of Botryosphaeriaceae family, *N. australe*, *N. luteum*, *N. parvum* were reported to be involved in the branch canker disease complex on avocado trees [52]. Others diseases like gummosis of mango [53], stem dieback of blueberries [54] and canker of *Juglans regia* seedlings [55] were caused by *N. parvum*.

Pathogenicity tests showed that *N. parvum* isolate was pathogenic towards strawberry leaves. The pathogen inoculated plants exhibited disease symptoms after one week, while un-inoculated leaves were without such symptoms. In Morocco, the present study confirms the capability of *N. parvum* to induce lesions on strawberry leaves. Thus, strawberry plants face a new threat and emergent pathogen which may be ignored on account of the difficulties of field diagnosis of the causal organism and the lack of knowledge about the environmental conditions, the thermal, humidity and time requirements necessary for mycelial growth, spore germination and pycnidia production. According to Espinoza et al. [40], symptoms caused by Botryosphaeriaceae spp. resemble those caused by *Pestalotiopsis* and *Phomopsis* spp., which sometimes coexist in the same plant.

It is recommended that the frequency of this fungal species should be monitored to determine development, spread in other areas and colonize others crops which can be more susceptible.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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