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PATHOGENICITY OF *COLLETOTRICHUM LUPINI* ON DETACHED LEAVES AND STRAWBERRIES OF *FRAGARIA ANANASSA* IN MOROCCO

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Abstract

Strawberry cultivation is one of the most important fruit crops worldwide, but it is constantly threatened by fungal pathogens. Among them, *Colletotrichum* spp poses a serious threat to strawberry production. In this study, a pathogenic characterization of one isolate of *Colletotrichum lupini* isolated from crown of senescent strawberry plants and identified for the first time in Morocco was tested using two inoculation methods. For the first one, using the mycelial disc of *C*. *lupini*, the result showed that without injury, the severity index was ranged between 62.51% and 72% compared to 57.40 and 77.83% in wounded leaves. The conidia production reached 76173.48 conidia.cm^{-2}. On strawberries, the size of lesions was uneven measuring 14.1 mm using agar disc's and 21.6 mm for inoculation by conidial suspension. The Re-isolation of the original isolate was successful which fulfilling Koch's postulates. Thus, the pathogenic capacity of *Colletotrichum lupini* to cause anthracnose disease on leaves and strawberries of *Fragaria ananassa* was confirmed. Further pathogenicity analysis on strawberry plant is required under greenhouse and field scale.

Keywords: Strawberries, Leaves, Anthracnosis, Pathogenicity, Morocco.

INTRODUCTION

Anthracnose is one of the most damaging fungal diseases that hampers strawberry production and yield where strawberries are grown [1]. The causal agents include *Colletotrichum* species which can infect various strawberry tissues, causing black spots or irregular spots on leaves, sunken black spots or necrosis lesions on petioles, stolons, and fruits, and wilting of the whole plant due to crown rot [2]. During warm, rainy, or humid weather, anthracnose can cause the death of up to 80% of seedlings in the nursery and yield losses of over 50% in the field [2, 3]. Up to 30 and 40% plant loss during the seedling stage and ~20% after transplanting are caused by anthracnose crown rot (ACR) [4].

Indeed, *C. gloeosporioides* and *C. acutatum* species are the most prevalent agent of anthracnose with *C*. *fragariae* in strawberry plants [5] in China. In Morocco, *C. gloeosporioides* and *C. acutatum* were previously mentioned in strawberry fields [6-7] and some isolates were demonstrated to be the causal agents of strawberry anthracnose [8]. Many other new species of Colletotrichum genus like *C. karstii* [9] and *C. miaoliense* [10] were recognized to be infectious agents of strawberry anthracnose in Taiwan. Additionally, *C. fructicola*, *C. siamense*, and *C. nymphaea* [11] were also be known to cause leaf and crown anthracnose in strawberry plants [12]. According to Zhang er al. [14], this disease is associated with a diversity of pathogenic fungi including *Colletotrichum siamense*, *C. fructicola*, *Fusarium oxysporum*, *F. commune*, *F. equiseti*, *F*. *solani*, *F. tricinctum*, *Epicoccum sorghinum*, *Stemphylium*

lycopersici, *Clonostachys rosea*, *Phoma herbarum*, and *Curvularia trifolii* which exhibited different degree of virulence on strawberry plants. Recently, the review focused on providing an accurate inventory of Colletotrichum species pathogenic to strawberry via revisiting the discovery history scrupulously over 90 years revealed a total of 23 Colletotrichum species clustered into five species complexes and two singleton taxa were accepted as strawberry pathogens with global occurrence [15].

In Morocco, another species was molecularly characterized and identified as *Colletotrichum lupini*. It was newly isolated from strawberry plants showing seedling death [16]. Thus, considering the relevance of this sector and the susceptibility of these crops to several pathogens, it is important to provide a correct pest diagnosis for an accurate and effective disease management. Therefore, we aimed to examine the pathogenicity of *C*. *lupini* isolate and to highlight the fungal community in association with infected strawberry plants. In this regard, a mycological analysis was performed then a Koch's postulates was done to view the pathogenicity that *Colletotrichum lupini* manifested.

MATERIALS AND METHODS

Plant Material

Healthy leaves and strawberries were harvested from two months-year-old potted bare root strawberry plants of Fortuna variety and maintained in greenhouse at the Kenitra Unversity.

Fungal Material

One representative isolate of *Colletotrichum lupini* (FCollu 3) was tested. The GenBank accession number for the nucleotide sequence of this isolate is MN064855 voucher RAB107306 as was reported by El Alaoui et al. [16]. This isolate was isolated from crown of camarosa variety grown in Dlalha farms at Moulay Bousselham (Morocco) province.

Inoculum Preparation

The *Colletotrichum lupini* isolate (FCollu 3) was cultured on potato saccharose agar (PSA). Plates with sporulating cultures were flooded with 10 mL of sterile distilled water and drop of Tween 20 then conidia were dislodged by gently scraping the colony surface a fine meshcloth to remove mycelial fragments, and spore concentration was adjusted to 1×10^5 with a Malassez slide.

On Detached Leaves

The pathogenicity assay on detached leaves were established both on wounded and unwounded leaves. Leaves were wounded at the center of the adaxial surface using a sterile needle. Healthy leaves were washed with tap water, surface-sterilized with 5% sodium hypochlorite for 1 min, rinsed once with sterile water, and finally air-dried on sterilized filter paper.

For the first inoculation methods, leaves were inoculated across its upper surface, with mycelial plugs (5 mm diameter) taken from the edge of a 1-week-old colony of *C. lupini*. Non-infested PSA plugs (5 mm diameter) were used for controls. For the second method, leaves were sprayed with conidial suspension (final concentration of 10^6 conidia/mL) added with a drop of Tween 20.

These leaves were placed in 9-cm-diameter Petri dishes containing 2 filter paper discs moistened with sterile distilled water. Then, plates were kept in a growth chamber at 25° C with a 12 h photoperiod for 7 days. The experiment was conducted in two independent trials, each consisting of six leaves per isolate. The six inoculated leaves were incubated in a moist chamber overnight at 25°C in the dark, then for 7 days at 25°C under a photoperiod of 12 h of light and 12 h of darkness.

Strawberries Pathogenicity Assay

In the first inoculation method, six healthy cv. Fortuna fruits were wounded with a sterile blade (a cut 2-mm deep and 4-mm long). Next, a 4 mm-diameter culture agar plug cut from the margins of 7- day-old Colletotrichum isolate colony was placed in each of two spaced wounds [22]. Following inoculation, the fruits were incubated at room temperature in a moist chamber with 98% RH until symptoms were observed (2–4 days).

In the second inoculation method six healthy cv. Fortuna strawberries were wounded with a sterile blade (a cut 2-mm deep and 4-mm long). Next, 10 µl of the suspension was pipetted onto the wounds (Guetsky et al., 2005). Sterile water was applied to control plants. Following inoculation, the fruit boxes were incubated at incubator in the darkness at 22–25°C until symptoms were observed (2–4 days).

The fungus was re-isolated from symptomatic leaves

Disease Leaves Scoring

The diseased leaf area was scored 15 days after inoculation using the scale of Stover modified by Gauhl et al (1995) [23]: 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 (IS) of the disease was calculated using the formula: IS= (Σnb/ (N – 1) x T) ×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.

Sporulation upon Leaves

The conidia production (Conidia. cm^{-2}) of *Colletotrichum lupini* isolate (FCollu3) on the inoculated strawberry leaves was estimated according to the technique of [23]. Ten days later, the leaves showing lesions were cut into pieces of 1 $cm^2$and placed in 90 mm Petri dishes on three filter paper discs humidified with sterilized distilled water. The dishes were incubated for 48 hours under continuous fluorescent lighting.

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

Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) and LSD test at 5% level.

RESULTS AND DISCUSSION

Almost all inoculated leaves manifested brown lesions in wounded leaves. Uninjured leaves of strawberry cv. Fortuna sprayed by a spore suspension underwent slow or no disease occurrence showing a circular to ovoid spots dark brownish to black colored which measured 0.4 to 1.2 cm in size at 7 post inoculation days. From 10 dpi, leaves became depressed, deformed and desiccated. Lesions coalesced along time causing leaves-wilting and blight (Figure 4). *C*. *lupini* was consistently reisolated at 14 days post inoculation (Figure 1).

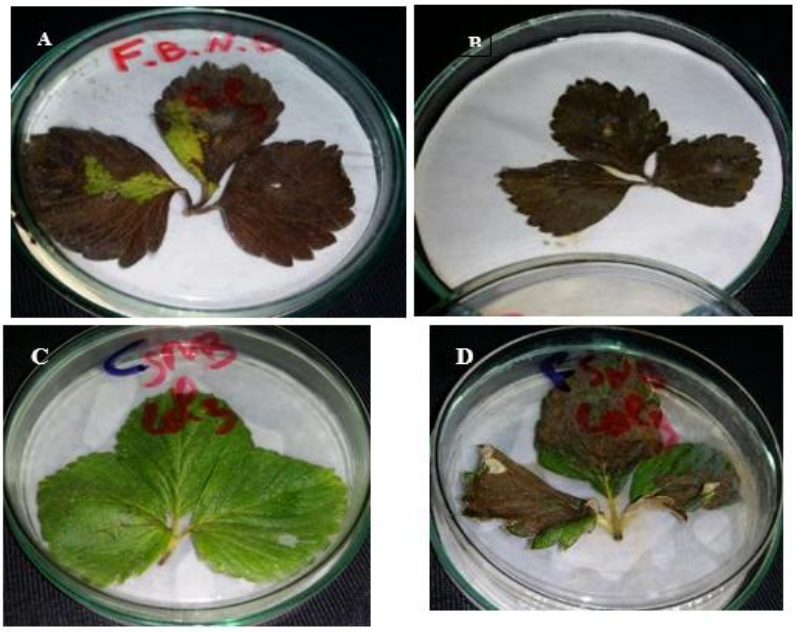

Figure 1: **Strawberry leaves of Fortuna variety with anthracnose symptoms caused by *Colletotrichum lupini* isolate after 7 days inoculated with spore suspension or 5 mm culture disc. (A-B) Symptoms of Inoculation with culture disc on wounded leaves (A) and unwounded leaves (B); C Control leaves; (D) Anthracnose symptoms after inoculation with spore suspension.**

Disease severity index was significant regardless the inoculation method used. They varied between 57 .4%-77.83% and 83.51-100% respectively after 7 and 14 days from inoculation day.

Table 1: Severity indexes and lesion dimensions on leaves of Fortuna variety 7 and 14 days after inoculation with *Colletotrichum lupini* isolate.

Inoculation Method		Severity index on leaves		Conidia production
		Incubation time		(conidia/cm^2)
		7 days	14 days	
Mycelial plug	Non injured	62.51^c	100^a	58289.08^c
	Wounded	57.40^d	96.29^b	61690.13^b
Spore suspension	Wounded	77.83^a	87.03^c	76173.24^a
	Non injured	72.00^b	83.51^d	54722.448^d

Values in the same column followed by the same letter are not significantly different at the 5% level (LSD)

Colletotrichum lupini isolate (FCollu3) was also able to produce conidia on leaves whose number ranged between 54722, 44 and 76173, 48 conidia/cm^2 according to inoculation method.

On detached strawberries

Three days after inoculation, the stored strawberries, showed rot symptoms, brown lesion affecting their firmness and superficial tissues. The infection was expressed as sunken brown to black lesions appeared around the wound area of the strawberries. At 7 days post inoculation, whitish to pale pink fine mycelia was visible then subsequent salmon to orange fruiting bodies (acervuli) of the pathogen are formed on the lesions. Whereas control fruits remained disease-free (Figure 2).

Figure 2: Strawberry fruit rots induced by *Colletotrichum lupini* isolate at 7 days post-inoculation. (A): Inoculation by drop of conidial suspension; (B):Inoculation by infested agar disc; (C):Control strawberry sprayed with drop of sterilized distilled water; (D): Control strawberry sprayed with sterilized distilled water.

The mean diameter of necrotic lesions induced by artificial inoculation of strawberries recieving 5 mm mycelial disc of *C*. *lupini* were in the order of 16.4 mm, 14.1 mm in unwounded ones, while the lesions were more extent with conidial suspension droplets attaining 21.6 mm and 20 mm in wounded and un-wounded strawberries respectively (Table 2).

Table 2: Lesion Dimensions on Strawberries of Fortuna variety 5 days after inoculation with *Colletotrichum lupini* isolate.

Inoculation Method		Mean lesion diameter on strawberries (mm)
Mycelial plug	Non injured	14.1^{d}
	Wounded	16.4^{c}
Spore suspension	Wounded	21.6^{a}
	Non injured	20^{b}

Values in the same column followed by the same letter are not significantly different at the 5% level (LSD)

On broad host range, *Colletotrichum* sp. is the causative agent of crown rot [24], fruit decay [25], root necrosis [3], black spots on leaves [26] and upon petioles and stolons [27]. *C. acutatum* has been detected on strawberry planting materials in Switzerland [28], and Belgium [29]. Planting material that is latently infected with *C. acutatum* is believed to be the main inoculum source [30, 31, 32]. The work results of [33] report the latent entry and spread of *Colletotrichum acutatum* (species complex) in strawberry fields.

In Morocco, *Colletotrichum* sp., is common fungal contaminants of strawberry plants [6, 7]. El Kaissoumi et al., [8] have demonstrated the virulence of *C. gloeosporioides* and *C. acutatum* inducing anthracnose symptoms in strawberry plants. Species of Colletotrichum affect a wide range of plants, often causing diseases known as anthracnose, on many fields and horticultural crops [34].

A new fungal agent belonging to Colletotrichum species complex was recovered from strawberry plants. Based on molecular traits and colony morphology, the characterization of *Colletotrichum lupini* within this complex was achieved and a new host for *C. lupini* has therefore been determined [16]. Indeed, *Colletotrichum lupini* belongs to the *C*. *acutatum* species complex [36]. It is the causal agent of anthracnose, the most important disease in lupin cultivation worldwide [37]. In Morocco, it was reported for the first time as the causal agent of anthracnose on olive fruits (Msairi et al., 2020) [38] by inducing fruit rot and lesions on leaves. According to these authors, the majority of the *C. lupini* isolates collected in Europe, Australia, South Africa, the USA and Chile belong to the highly virulent genetic group II [39, 40], On lupin, the most distinctive symptoms stem twisting, necrotic lesions on stems, pods, petioles with elliptical, dark brown, and sunken lesions, which were often completely girdled by these lesions, resulting in wilting and death of the leaves [41, 42]. *C. lupini* has a hemibiotrophic lifestyle [42], colonizing the host endophytically and causing the typical disease symptoms of stem and pod [43]. This is followed by the formation of necrotic lesions containing orange masses of conidia (acervuli) when dispersed within the crop, leading to secondary infections [44, 45]. In line with these conclusions, our *C. lupini* isolate was able to affect leaf tissues inducing extended necrotic lesions before producing secondary inoculum. In contrast, [26] described a black leaf spot phase of strawberry caused by *C. gloeosporioides* which is often found in association with anthracnose symptoms on runners and petioles.

Through leaf-wound inoculation, superior severity index and sporulation were recorded. According to [42], stem-wound inoculation assay was ascribed to be highly reproducible and strongly correlated to field performance under natural infection pressure. The infective potential of our isolate was also confirmed on strawberries, producing irregular, brown decay lesions around the wound areas covered gradually with pinkish-orange spore mass that gain the whole surface of strawberries.

De Silva et al., 2021 [46] have demonstrated that pathogenicity of Colletotrichum on resistant host *Capsicum chinense* PBC932 was dependent on the inoculation method and the maturity stage of the fruit. This was in line with our findings which demonstrate that wounding in healthy leaves greatly enhanced the pathogenicity of this species to strawberry leaves cv. Fortuna; At the same manner, the inoculation methods significantly affected the size of lesion appearing after artificial inoculation.

CONCLUSION

As shown above, our C. *lupini* isolate had proved its pathogenicity on leaves and strawberry fruit which clearly not concordant with the fact that *C*. *lupini* is preferentially the lupin anthracnose pathogen [38]. We can also admit that it had a strong implication in seedling death and strawberry plants collapsing after transplanting. Finally, as far as we know, this is the first report of pathogenic capacity of *C. lupini* affecting strawberry leaves and fruit in Morocco. Future studies will focus on detailed experiments to deeply investigate pathogenicity and host specificity.

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