

# Biology and Physiology of *Colletotrichum acutatum* Strains Causing Strawberry's Anthracnose

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## Abstract

Seven *Colletotrichum acutatum* strains isolated from strawberries were cultivated on various culture media and tested *in vitro* and *in vivo* on *Fragaria x ananassa* for anthracnose symptoms. PDA caused an optimum growth of all isolates, MEA, ML and Strawberry allowed a good but not optimal growth. Czapeck, Sabouraud and the organic medium from potato gave the lowest growth rate of all isolates. PDA allowed a good sporulation of isolates follow-up by strawberry, MEA; in contrast, ML, Czapeck and Sabouraud gave a low sporulation. The fungal development is maximal at 25°C and 27°C for all the isolates studied. No growth was observed at 5°C and 37°C. The studied strains developed at all pH values. They didn't develop at 0.6, 0.65 and 0.7 aw but mycelial growth was perfect at 1 and 0.95 aw. The *in vitro* test of the pathogenicity caused by *C. acutatum* strains on strawberry's leaves showed an increasing percentage of infection with time and different infection rates among *C. acutatum* strains, strain Ca6 having a pathogenic power very high compared to the other isolates. After *in vivo* inoculation and incubation of the seedlings, all isolates caused severe symptoms related to anthracnose on leaflets and petioles of the studied strawberry plants.

## Keywords

Anthracnose, Strawberry, *Colletotrichum acutatum*, Environmental Factors, Pathogenicity

## 1. Introduction

Strawberry plant, *Fragaria x ananassa*, is one of the most widespread horticultural crop in the world. In Morocco, it was first introduced in the fifties, but its culture started toward the end of the seventies in two irrigated perimeters of the Loukkos and Souss-Massa. Ten years later the culture extended to the perimeter of the Gharb and then the area of Souss [1]. The climate in these regions allows the growth of strawberry in greenhouses but commercial fields are ideal for the development of a large number of diseases like anthracnose. The latter is regarded as an economically important disease affecting different hosts in the world caused by different species of *Colletotrichum* [2] [3]. Three species have been reported as causal agents of strawberry's anthracnose: *C. acutatum* J. H. Simmonds, *C. gloeosporioides* (Penz.) Penz. & Sacc., and *C. fragariae* A. N. Brooks [4] [5].

*C. gloeosporioides* and *C. acutatum* are distributed on a large number of hosts in the world, whereas *C. fragariae* has a range of hosts very close [6]. Anthracnose causes up to 80% of death in nurseries and more than 50% loss of performance in the strawberry fields [7]. It has been defined as one of the most serious diseases in the commercial production of strawberry fruit. *Colletotrichum acutatum* is the most frequently species reported of the genus. It is today known as especially destructive on strawberry fruit [8]. It causes mainly black spots on fruit and can also attack crowns, roots and leaves [9] [10] [11].

Environmental factors such as relative humidity, pH and temperature have been reported to have a profound influence on the virulence of a variety of fungi. The optimum temperatures for growth were often found between 25°C and 30°C; because of high temperatures the mycelial growth become weak and, in some cases, the mortality of fungi can occur. Several studies have been conducted on the effects of environmental factors on the growth of *C. acutatum* [12].

In this context, the aim of this study was to examine the behavior of seven isolates of *Colletotrichum acutatum* by changing the medium composition, temperature, pH and water activity and to study the disease severity caused by these isolates on *Fragaria x ananassa* *in vitro* and *in vivo*.

## 2. Materials and Methods

### 2.1. Fungal Material

Seven isolates of *Colletotrichum acutatum* (Ca1, Ca2, Ca3, Ca4, Ca5, Ca6 and Ca7) have been isolated from strawberry plants which has been collected from strawberry's fields of Loukkos (Larache, Morocco) naturally affected by anthracnose, purified in Laboratory of Plant Biotechnology, Faculty of Sciences, Tetouan. They have been cultivated on PDA (Potato Dextrose Agar) medium for 7 to 10 days at 25°C in the dark, and successive subculturing were made up within a total purification of strains.

Their identification has been carried out by macroscopic and microscopic observations with the help of determination keys [11] [13].

## 2.2. Study of the Pathogenicity of the Isolates of *Colletotrichum acutatum*

### 2.2.1. Plant Material

Fourteen strawberry plants (*Fragaria x ananassa* (Weston)) Duchesne ex Rozier cultivar Camarosa (**Figure 1**) were conducted at the Laboratory of the Plant Biotechnology in Faculty of Sciences of Tetouan in pots containing black sandy soils from the origin field of the plants. The plants were watered daily.

### 2.2.2. Preparation of the Inoculum

Conidial suspensions of the isolates were obtained by adding 10 ml sterile distilled water on Petri dishes containing 10 to 15 day culture of *C. acutatum* on PDA, and gently rubbing the colonies using a sterile Pasteur pipette. At the end of spore release, the suspension of spores was filtered using sterile gauze nylon, pore size 100  $\mu\text{m}$ . Concentration was determined using a Malassez cell.

### 2.2.3. *In Vitro* Leaves Inoculation

Young and healthy leaflets were harvested, carefully washed with distilled water and disinfected with a solution of sodium hypochlorite (2% w/v) for five minutes followed by three washes of 15 minutes in sterile distilled water. Leaves have been dried under an air stream in laminar flow hood. They have been filed on sterile Petri dishes (four leaflets per box) containing a double layer of sterile filter paper previously soaked in sterile distilled water. Central lesions were incised with a sterile scalpel in each leaflet [14]. Three Petri dishes per strain were used.

On each lesion 30  $\mu\text{l}$  of *C. acutatum*'s suspension ( $10^4$  spores/ml) was added (**Figure 2**); Petri dishes were incubated in dark at ambient temperature. As a blank, leaflets were inoculated by 30  $\mu\text{l}$  sterile distilled water. The test was repeated three times.



**Figure 1.** Strawberry plant (cultivar Camarosa).



**Figure 2.** Strawberry leaflets inoculated with one of *Colletotrichum acutatum* strains.

The pathogenicity was recorded on the following scale:

0 = no visible disease symptom;

1 = less than 15% of leaflet's surface is infected;

2 = 15% - 35% of leaflet's surface is infected;

3 = 36% - 49% of leaflet's surface is infected;

4 = 50% - 74% of leaflet's surface is infected;

5 = more than 75% of leaflet's surface is infected.

The percent of pathogenicity was calculated by the formula bellow (1):

$$\text{PDI} = \frac{\text{Sum of all numeric notes}}{\text{Total leaflet's surface} \times \text{maximale note}} \times 100 \quad (1)$$

#### 2.2.4. Inoculation *in Vivo*

Inoculation of plants was made by spraying 300 ml of conidial suspension of each tested pathogen (1 pot/strain). Plants were then covered for 48 hours with black plastic bags sprayed inside with sterile water to maintain a high relative humidity, necessary for germination and direct penetration of conidia (without injury). Pots were subsequently transferred in greenhouse (temperature ranging between 25°C to 28°C) in photoperiod. The percent of the pathogenicity degree in petioles (PDP) and leaflets (PDL) was calculated by (2) (3):

$$\% \text{PDP} = \frac{\text{Number of infected petioles}}{\text{Total number of petioles}} \times 100 \quad (2)$$

$$\% \text{PDL} = \frac{\text{Number of infected leaflets}}{\text{Total number of leaflets}} \times 100 \quad (3)$$

### 2.3. Growth and Sporulation of the Isolates of *Colletotrichum acutatum* on Culture Media

The isolates of *Colletotrichum acutatum* have been grown on four mixed culture media (Potato Dextrose Agar (PDA), Yeast Malt (ML), Sabouraud and Malt Extract Agar (MEA)), a semi-synthetic (Czapek) and two organic culture media

(Strawberry and Potato (400 g of organic matter + 16 g of agar in 1000 ml of distilled water)). Growth and sporulation of each isolate were studied.

#### 2.4. Effect of Temperature on Mycelial Growth, Germination and Sporulation of the Isolates of *Colletotrichum acutatum*

Mycelial discs, from older cultures of ten days, were placed in Petri dishes of 70 mm containing the culture medium PDA. These plates were incubated in the dark at different temperatures (5°C, 18°C, 23°C, 25°C, 27°C, 30°C and 37°C).

#### 2.5. Effect of pH on Mycelial Growth, Germination and Sporulation of the Isolates of *Colletotrichum acutatum*

The effect of pH on the development of *Colletotrichum acutatum* strains was tested at pH 4, 4.5, 5, 5.5, 6, 6.5, 7.5 and 8. The culture media PDA has been stamped, according to the desired pH, by different buffers (Table 1) [15]. Using a pH-meter, the pH was adjusted by addition of HCl and NaOH 1N [15]. Plates were incubated at 27°C in the dark.

#### 2.6 Influence of the Water Activity on the Development of Seven Isolates of *Colletotrichum acutatum*

The water activity ( $a_w$ ) represents the availability in open water for the biochemical reactions for the development of microorganisms. Different values of activity of the water have been tested (1; 0.95; 0.90; 0.85; 0.80; 0.75; 0.70; 0.65 and 0.60) by the addition of glycerol in the culture medium PDA [15] [16], which will attach a part of the water and make it unusable to microorganisms.

#### 2.7. The Studied Factors Were Tested on

##### 2.7.1. The Mycelial Growth

For each medium, three Petri dishes were inoculated in their center by mycelial discs of 5mm diameter. After ten-day incubation at 25°C in the dark, the growth

**Table 1.** Different values and buffers of used pH.

pH	Buffer		Molecular weight	20 mM (g/l)
	Common	Chemical		
4	Trizma (TrisHCl)	2-amino-2-(hydroxymethyl)-1,3-propanediol, hydrochloride	157.59	3.152
4.5				
5				
5.5	MES	2-( <i>N</i> -morpholino)ethanesulfonic acid	213.25	4.265
6				
6.5	PIPES	piperazine- <i>N,N'</i> -bis (2-ethanesulfonic acid)	302.4	6.048
7.5	MOPS	3-( <i>N</i> -morpholino)propanesulfonic acid	209.3	4.186
8	BICINE	<i>N,N</i> -bis(2-hydroxyethyl)glycine	163.2	3.264

has been recorded by measuring the mycelial growth rate ( $G$ ) (4)

$$G = \frac{\text{Sum of 2 perpendicular diameters}}{2} \quad (4)$$

### 2.7.2. The Sporulation

It was evaluated with the help of a Malassez cell. Conidia were obtained from ten-day cultures by placing 2 discs in a 5 ml tube containing 2 ml sterile distilled water and agitation on a vortex.

### 2.7.3. The Germination

30  $\mu$ l of a suspension of  $10^4$  spores/ml was spread on the surface of Petri dishes containing 0.5 g agar/100 ml distilled water. The counting of the germinated spores was carried out on a total of 100 spores after 24-hour incubation at 25°C. In each test, three Petri dishes were used and the experiment was repeated three times.

## 3. Statistical Analysis

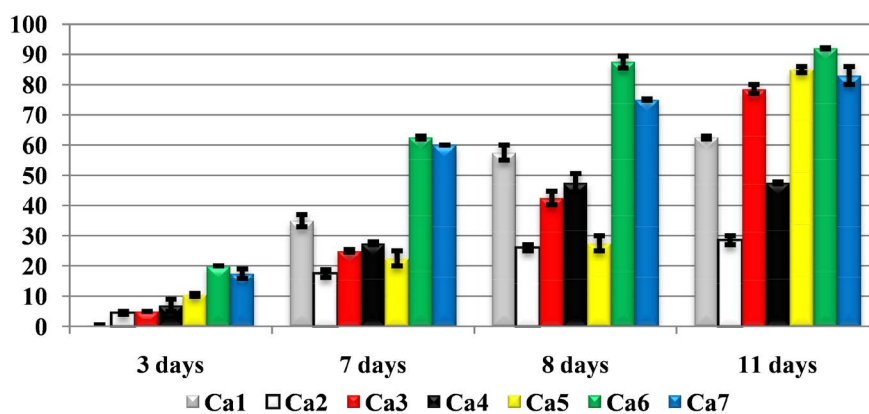
Before you begin to format your paper, Isolates development rates have been subjected to analysis of variance (ANOVA) using STATISTICA software for Windows V.6. The statistical significance of the results was determined by performing a test of Duncan's multiple range ( $p < 0.05$ ).

## 4. Results

### 4.1. Pathogenicity of Isolates of *Colletotrichum acutatum*

#### 4.1.1. In Vitro

The percentage of infection on strawberries leaves increases with time and there is a difference in the rate of infection among different isolates (Figure 3). Strain Ca1 in the first 3 days does not show any spot on the leaves against the other strains where the percentages of infection vary between 5 (Ca2, Ca3 and Ca4) and 20% (Ca6). Strain Ca6 has a pathogenic power very high compared to the

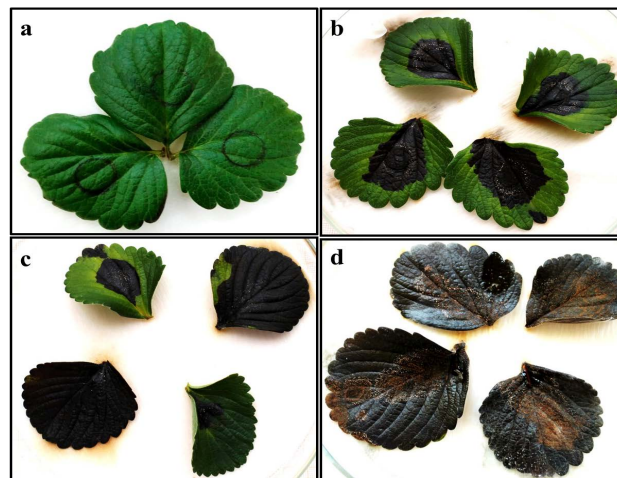


**Figure 3.** Pathogenicity degree of seven isolates of *Colletotrichum acutatum* on the leaflets of strawberry *in vitro*.

other isolates. After 11-day incubation, strain Ca6 reached up to 95% infection, all inoculated leaves being infected (**Figure 3** and **Figure 4**). All leaflets inoculated with isolates showed symptoms typical of anthracnose symptoms with black spots at the surface of the leaflets (**Figure 4**).

#### 4.1.2. *In Vivo*

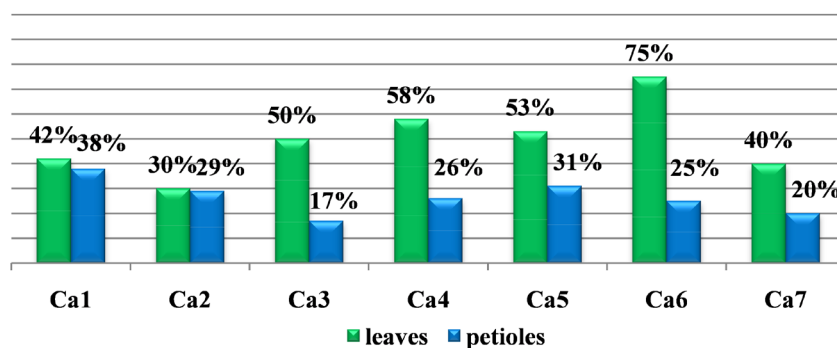
After the inoculation and incubation of the seedlings, all the isolates cause severe symptoms typical from anthracnose on leaflets and petioles. The severities of the disease for each isolate increase in the course of time. Isolate Ca6 is the most aggressive among other isolates on leaflets and petioles (**Figures 5-7**).



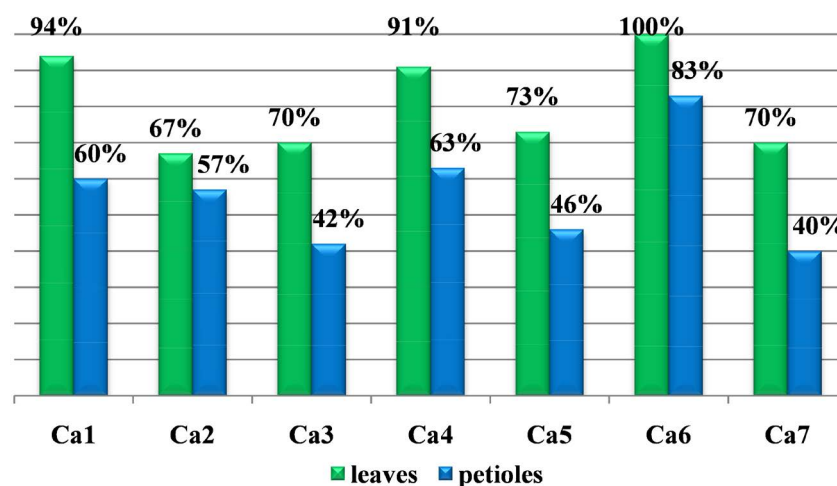
**Figure 4.** Development of the degree of the pathogenicity of the strain Ca6 on leaves: (a) inoculated leaflet by the suspension at the day of inoculation; (b) infected leaflets after 3 days; (c) infected leaflets after 8 days; (d) infected leaflets after 11 days of incubation.



**Figure 5.** Symptoms of anthracnose caused by strains Ca6 on petioles and leaves of strawberry “Camarosa” compared with blank (c).



**Figure 6.** Pathogenicity degree of seven *Colletotrichum acutatum* strains on leaflets and petioles of strawberry “Camarossa” *in vivo* after 8 day-incubation.



**Figure 7.** Pathogenicity degree of seven isolates of *Colletotrichum acutatum* on the leaflets and petioles of strawberry “Camarossa” grown *in vivo* after 20 day-incubation.

#### 4.2. Effect of Culture Medium

All culture media have enabled the mycelial growth of *Colletotrichum acutatum* (Figure 8) with different means of development (Table 2). PDA remains the culture medium the most favorable for the mycelial growth of *Colletotrichum acutatum*, with a maximum enlargement of all isolates. MEA, ML and Strawberry have allowed a good growth but not optimal. Czapeck and Sabouraud have given an average growth of all isolates.

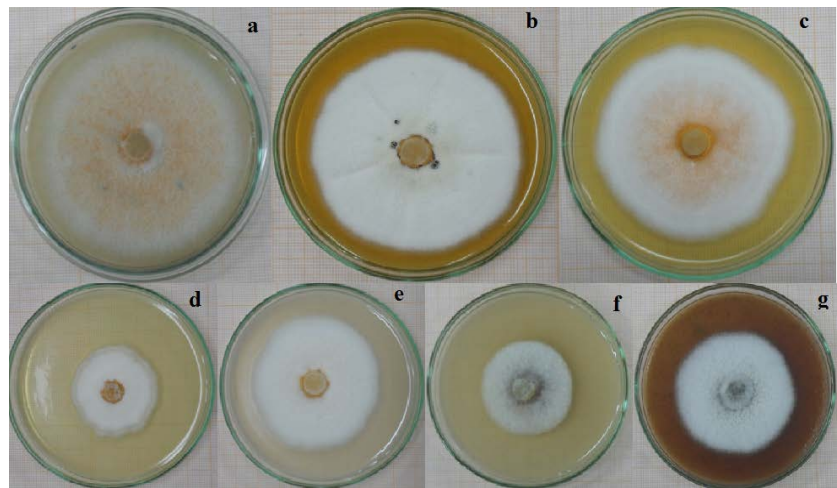
PDA has allowed a good sporulation of isolates follow-up by Strawberry, MEA while ML, Czapeck and Sabouraud have allowed low sporulation (Table 2).

#### 4.3. Effect of Temperature

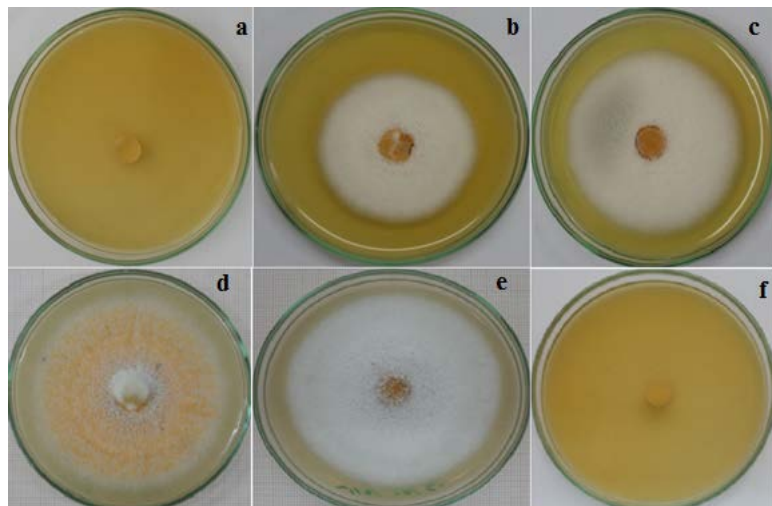
All isolates didn't develop at 5°C and 37°C (Figures 9-12). Mycelial growth is low at 30°C, average at 18°C, maximum at 25°C and 27°C for all the isolates studied (Figure 10).

The seven isolates sporulate weakly at 18°C and 3°C. Sporulation is maximal at 25°C and 27°C and averages 23°C for all the isolates studied (Figure 11).

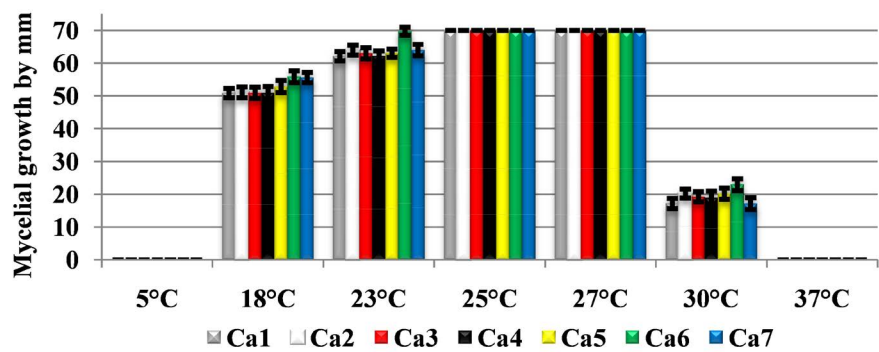




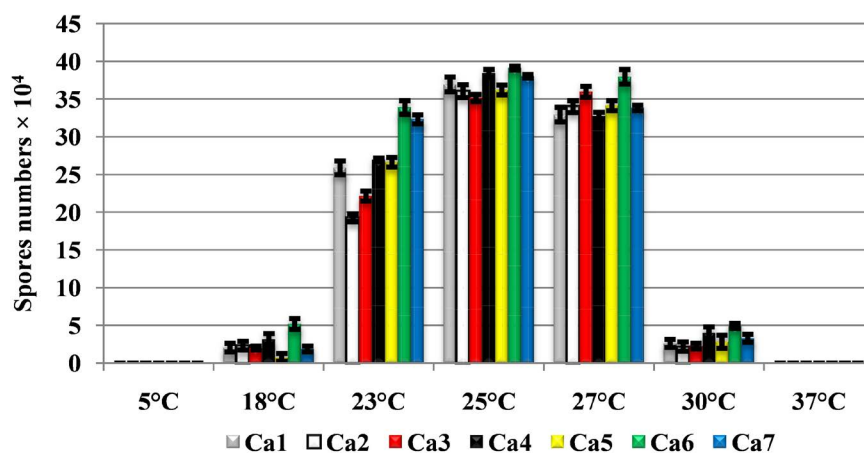
**Figure 8.** Effect of culture media: (a) PDA; (b) ML; (c) MEA; (d) Sabouraud; (e) Czapeck; (f) Potato and (g) Strawberry on the mycelial growth of seven isolates of *Colletotrichum acutatum* after 10 days of incubation.



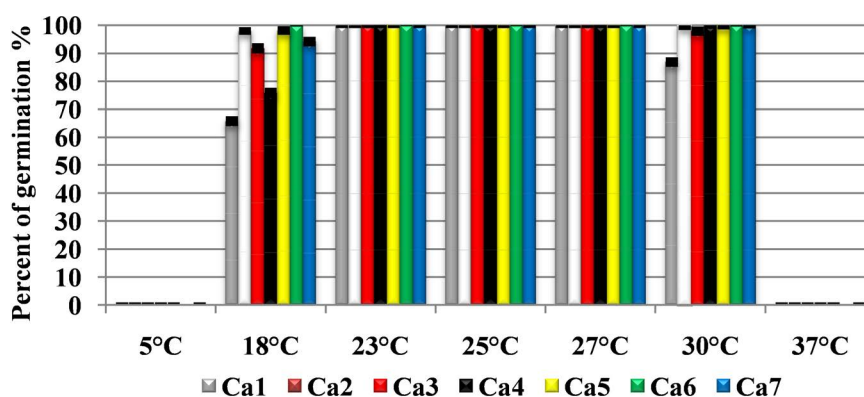
**Figure 9.** Influence of the temperature at: (a) 5°C; (b) 18°C; (c) 23°C; (d) 25°C; (e) 27°C and (f) 37°C on the mycelial growth, on PDA of seven isolates of *Colletotrichum acutatum* after 10 days of incubation.



**Figure 10.** Influence of the temperature on the mycelial growth (mm), on PDA of seven isolates of *Colletotrichum acutatum* after 10 days of incubation.



**Figure 11.** Influence of temperature on sporulation ( $\times 10^4$  spores/ml) of seven isolates of *Colletotrichum acutatum*.



**Figure 12.** Influence of the temperature on percent of germination of seven isolates of *Colletotrichum acutatum*.

**Table 2.** Effect of seven culture media on the mycelial growth (mm) after 10-day incubation and sporulation of *Colletotrichum acutatum*.

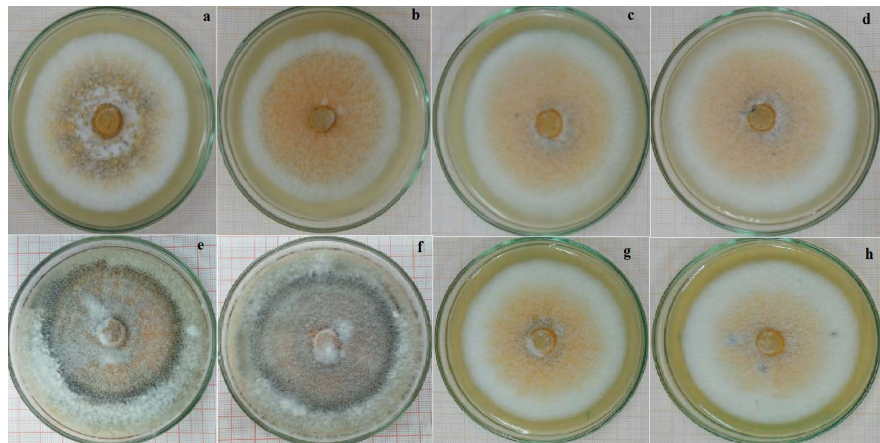
Culture medium	Mycelial growth (mm)	Sporulation ( $\times 10^4$ )
<b>Mixed medium</b>		
Potato Dextrose Agar (PDA)	70.00 $\pm$ 0.00 a	34.44 $\pm$ 0.86 a
Yeast Malt (ML)	66.31 $\pm$ 0.43 ab	19.03 $\pm$ 1.12 bc
Malt Extract Agar (MEA)	65.30 $\pm$ 0.93 ab	1.10 $\pm$ 0.29 d
Sabouraud	49.14 $\pm$ 0.47 bc	1.02 $\pm$ 0.18 d
<b>Semi-synthetic medium</b>		
Czapeck	55.67 $\pm$ 1.09 c	1.50 $\pm$ 0.32 d
<b>Organic medium</b>		
Strawberry	61.76 $\pm$ 0.98 b	26.78 $\pm$ 1.12 b
Potato	52.60 $\pm$ 0.43 bc	10.59 $\pm$ 0.62 c

Averages of a column with the same letter are not significantly different between them at the threshold of 5%.

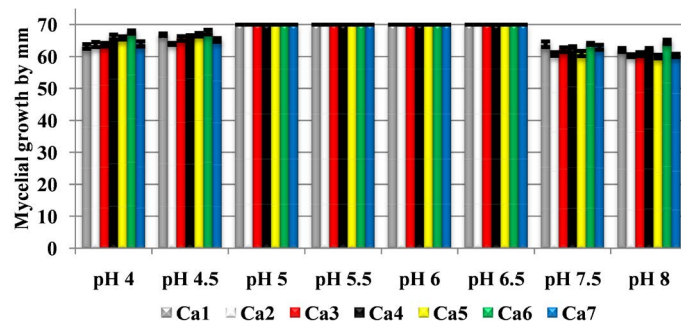
The conidia do not germinate at 5°C and 37°C. Conidial germination of all species studied is medium at 18°C and 30°C and maximal at 23°C, 25°C and 27°C (Figure 12).

#### 4.4. Effect of pH

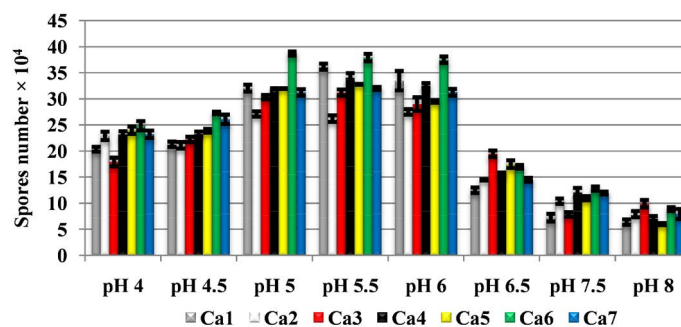
All isolates studied developed at all pH values (Figures 13-16). The mycelial growth is maximum at pH 5 and 5.5.



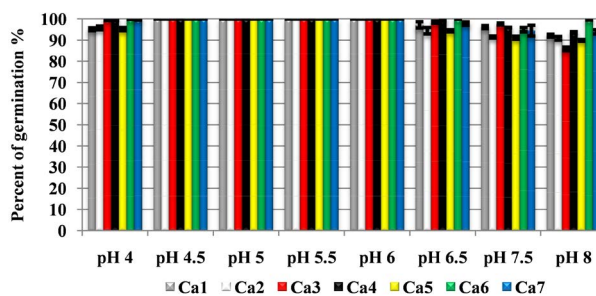
**Figure 13.** Influence of pH: (a) 4; (b) 4.5; (c) 5; (d) 5.5; (e) 6; (f) 6.5; (g) 7.5 and (h) 8 on the mycelial growth on the PDA of Ca6.



**Figure 14.** Influence of pH on the mycelial growth (mm) on PDA of seven isolates of *Colletotrichum acutatum*.



**Figure 15.** Influence of pH on sporulation ( $\times 10^4$  spores/ml) of seven *Colletotrichum acutatum* strains.

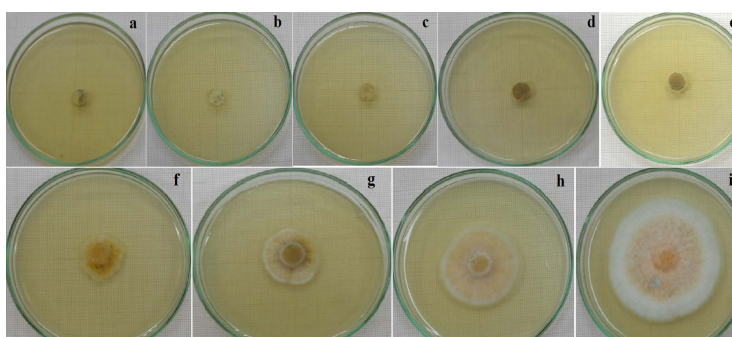


**Figure 16.** Influence of pH on the percent of germination of spores on water agar of seven isolates of *Colletotrichum acutatum*.

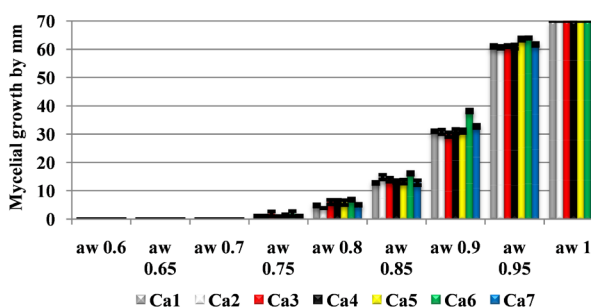
All isolates of *C. acutatum* germinated perfectly at pH = 4.5, 5, 5.5 and 6, and sporulated perfectly at pH 5, 5.5 and 6, moderately to pH 4 and 4.5 and weakly to pH 7.5 and 8.

#### 4.5. Effect of the Water Activity

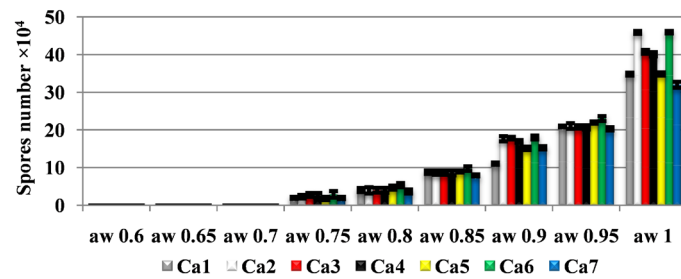
All isolates do not develop at aw 0.6, 0.65 and 0.7. The mycelial growth is perfect at aw 1 and 0.95, average at aw 0.85 and 0.90, and low at aw 0.75 and 0.8 (Figure 17 and Figure 18). Sporulation is perfect to aw 1, average at aw (0.9 and 0.95) and low at aw (0.75, 0.8 and 0.85) (Figure 19). The germination is maximal at aw 1, average at aw (0.9 and 0.95) and low at aw (0.75, 0.8 and 0.85) (Figure 20).



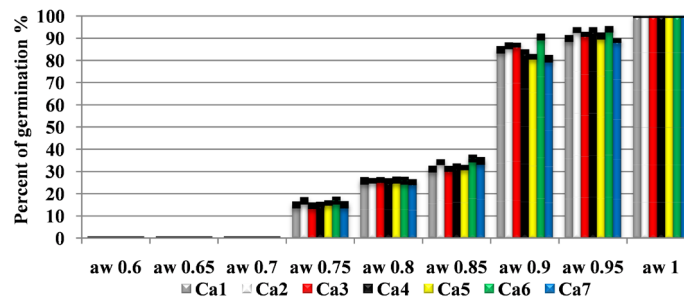
**Figure 17.** Influence of aw (a) 0.60; (b) 0.65; (c) 0.70; (d) 0.75; (e) 0.80; (f) 0.85; (g) 0.90; (h) 0.95 and (i) 1 on the mycelial growth of a *Colletotrichum acutatum* strain after 5 days of incubation.



**Figure 18.** Influence of water activity on the mycelial growth (mm) of seven *Colletotrichum acutatum* strains after 10 days of incubation.



**Figure 19.** Influence of water activity on sporulation ( $\times 10^4$  spores/ml) of seven *Colletotrichum acutatum* strains.



**Figure 20.** Influence of water activity on germination of seven *Colletotrichum acutatum* strains.

## 5. Discussion and Conclusion

*Colletotrichum acutatum* strains cause symptoms related to the anthracnose *in vitro* and *in vivo* but the severity of the disease varies from an isolate to another, isolate Ca6 representing a high level of aggressiveness among the strains studied; the percent of pathogenicity has been affected to 92% after 11 days of the *in vitro* inoculation and 100% after 20 days of the *in vivo* inoculation. Peres *et al.*, [17] have described that the symptoms caused by *C. acutatum* are mainly necrosis including burns on various types of tissues of the host such as leaves, petioles on a wide range of hosts. By comparing the pathogenicity of *C. acutatum* and *C. fragariae* on fruit, petioles and roots of *Fragaria x ananassa*, Tanaka *et al.*, [18] have found that *C. acutatum* is less aggressive than *C. fragariae* against this host. Same results were found by Smith and Black [11] and McInnes *et al.*, [19] who have noted the association of *C. acutatum* with lesions of the rhizomes. *C. acutatum* from strawberry can parasitize and cause diseases on other hosts [19] [20] [21] or, alternatively, survive on other cultures and on weeds without producing symptoms. Smith and Black [11], Peres *et al.*, [17] and Hyde *et al.*, [21] have found that their studied *Colletotrichum acutatum* isolates haven't caused leaf lesions on wounded inoculated leaves unlike two other *Colletotrichum* species whereas, in our study, all the isolates tested caused lesions on the strawberry leaves *in vitro*. *C. acutatum* isolated from almond and peach were demonstrated to be pathogenic on wounded and nonwounded fruit [22]; artificial inoculations demonstrated that fruits of all host species except for the banana were susceptible to *C. acutatum* isolates from strawberry [23].

Fungi generally require different pH and temperature conditions during the course of their development. These two factors influence the stages of their life cycle. The development of *Colletotrichum acutatum* changes with the environmental factors studied (culture medium composition, temperature, pH and aw).

Mycelial growth and sporulation of the strains are perfect in the culture medium PDA, followed by MEA and Strawberry. For ML, mycelial growth was good but the sporulation was very low as in the other culture media. PDA has been used as the base culture medium for the isolation, purification and growth of *Colletotrichum* species causal agents of grown strawberry's anthracnose [5] [11] [24] [25] [26]. Variation of temperature also affects the development of *C. acutatum* strains; 5°C and 37°C cause no development of the fungus. Germination of strain Ca4 is perfect at other temperatures; germination of Ca1, Ca2, Ca5, Ca6 and Ca7 is maximal at 23°C, 25°C, 27°C and 30°C. Sporulation of all isolates is too low at 18°C and 30°C, average at 23°C and maximum at 25°C and 27°C. Mycelial growth is maximum at 25°C and 27°C, average at 18°C and 30°C, and no growth is observed at 5°C and 37°C.

Miles *et al.*, [27], Grahovac *et al.*, [28] and Fernando *et al.*, [29] have found that *Colletotrichum acutatum* has an optimal development at 25°C ± 2°C. These results are consistent with other studies that have evaluated the effect of the temperature on species of *Colletotrichum* from different hosts [4] [12] [13] [30] [22]. The comparison of the effect of temperature between *C. acutatum* strains isolated from avocado, banana, guava, papaya, mango and passion fruit shows that the optimum temperature for their development is 28°C. None of the strains has grown at 8°C and only the isolates from avocado, papaya and banana have developed at 36°C [23]. Optimum germination of *C. acutatum* isolated from coffee has occurred at 21°C - 29°C [31] and its mycelial growth was maximal at 21°C [31] [32] [33].

The environmental pH plays an important role in the growth and differentiation of microorganisms. In the present work, pH variation has no remarkable effect on mycelial growth and germination of the seven strains studied: the mycelial growth, sporulation and germination of all strains reach an optimum at pH 5, 5.5, 6 and 6.5. *Colletotrichum musae*, the causal agent to anthracnose of banana tree, has an optimal development at pH 4.5 [34].

The water activity has a remarkable effect on the mycelial growth, which is optimal at 0.95 and 1, medium at 0.75 - 0.90 and null between 0.60 and 0.70: *C. acutatum*, the agent of anthracnose of strawberry, requires water in order to develop.

The anthracnose caused by several species of the genus *Colletotrichum* and especially *C. acutatum* represents one of the major fungal diseases of *Fragaria x ananassa*. The disease manifests itself by small circular spots which merge to form large elliptical spots on fruit and leaves. Therefore the control against this disease for cultures of strawberry plants, devoid of phytopathogenic agents, is necessary in order to produce healthy fruit to meet the consumer's requirement.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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