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Identification of *Fusarium oxysporum sf tracheiphilum* strains responsible of cowpea wilt in Far-north region of Cameroon

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ABSTRACT
Objective: The goal of the present study is to identify *Fusarium oxysporum sf tracheiphilum* strains causing cowpea wilt in the Far-north region of Cameroon and also to determine the more virulent strain.
Methodology and results: Isolation was performed by using the diseased plant tissues. Characterization of pathogens' strains was done by comparing cultural characteristics and microscopic features to previous studies. Pathogenicity test was done in a greenhouse on susceptible and resistant varieties in completely randomized design replicated thrice. Results showed the variability of mycelium's colours on PDA culture media. Conidia were produced on JOFF and MGA media while no sporulation was observed on PDA. All types of spores that characterized *Fusarium oxysporum* were observed. The reproduction of field symptoms was observed during the pathogenicity test. Strain 7 was identified as the more virulent.
Conclusion and application of findings: Fusarium cowpea wilt caused by *Fusarium oxysporum sf tracheiphilum* (FOT) is the main cause wilting and loss of cowpea on the field. This study findings can solve cowpea producers of the far-north region of Cameroon on their lack of knowledge on the aetiology of that disease though it is present in almost all farms. Strain 7 identified as the more virulent in the current work could be used by plant breeders for varietal screening for the resistance against the pathogen. In addition, further study is needed to determine the race of that strain.
Keywords: Fusarium wilt, sporulation, pathogenicity, cowpea, virulent

INTRODUCTION
In Cameroon, Cowpea is the third legume that is produced and consumed after peanuts and soybean (Agristat, 2012). The national production is estimated at 166 146 tons a year and 70% of that production is furnished by the only far-north region. It is harvested on a total area of 249 486 ha (Agristat, 2012). With his high level of seeds protein up to 40%, this

legume helps low income populations who can't get meat to solve the problem of protein deficiency in their diet (Dakora and Belane, 2019). Though demographic growth continuous and the cowpea seeds demand is constantly increasing in the market, seeds yield improvement remain low due to several constraints that affect this legumes among which diseases are one of majors problems. Cowpea wilt caused by *Fusarium* responsible of vascular wilting is one of the major disease constraint of this legume in field (Rodrigues and Maria, 2005). It is caused by *Fusarium oxysporum* f. s*p. Tracheiphilum* (Fot). The symptoms at the early stage of the disease start by slight chlorosis of the old leaves, which turn from green to yellow. This discoloration affects sometime just one side of the leaf or the plant. The progressing of the disease is characterized by defoliation, wilting which begins on one side, then the whole plant and later the dead of the plant (Englehard et Woltz, 1971; Ana *et al.,* 2020). Fusarium wilting disease is responsible of more than 50% of cowpea destruction on the field (Rodrigues and Maria, 2005) which classified this disease as the main cause of this legumes wilting. The disease of wilting due to *FOT* is widely distributed in the cowpea producers' countries like Nigeria, Australia, Brazil and the United States of America. The pathogen enters to the plant through roots and colonizes vascular system provoking then the wilting and the chlorosis of leaves (Pottorff *et al*., 2014). *Fusarium oxysporum* has an ability to live in soil during several years in absence of his host, generally in the form of chlamydospores which allows him to wait for the host to renew his development (Rejeti *and al*., 2014; Ana *and al.,* 2020**).** This species produce all types of spores namely macroconidia, microconidia and chlamydospores. The Conidia derived directly from hypha by mitotic division. The sexual form of the species is unknown (Gordon, 2017). Four races of *Fusarium oxysporum* f. s*p. Tracheiphilum* (*Fot1, Fot2, Fot3* and *Fot4)* were identified responsible of fusarium wilting (Armstrong et Armstrong, 1980; Swanson et Gundy, 1985; Ana *et al*., 2020). Although the pathogen has a high degree of specificity to his host at the level of strains called *forma specialis*, Race 1 (*Fot*1) was identified as also pathogen to soya (Armstrong et Armstrong, 1980; Swanson et Gundy, 1985; Ana *et al.,* 2020**)**. The capability of this pathogen to survive in soil make difficult its chemical control, economically not profitable and has a high risk on the environment and health. A farm management of the disease with crop rotation has a limited effect because the pathogen survives in the soil and then has ability to live without its host, which produces an unexpected results. The biological control based on the use of rhizobium (Ana *et al.,* 2020) is not known by producers and the biological fungicides are not present at local marked. Therefore, genetic control is more adequate. Previous works done in other countries like Nigeria and the United States of America (USA) have showed the existence of some resistant varieties against these fungi pathogens (Pottorff *et al.,* 2014; Omoigui *et al*., 2018). Unfortunately, results of these researchers revealed that resistant varieties are just for two or one race of the pathogen. It is then necessary to identify the more virulent strain pathogen in the locality where cowpea is cultivated for the best management of the disease. Thus, in Cameroon, despite the high diversity of cowpea cultivated in the far-north region, the identification of the pathogen responsible of cowpea *Fusarium* wilting is not yet done. The aim of the present study is to determine the strains of *Fusarium oxysporum* f. s*p. Tracheiphilum* causing the cowpea wilting disease in the far-north region in the perspective of the development of resistant varieties.

MATERIALS AND METHODS

Presentation of the zone of study: The study was carried out in fifteen villages of the far-north region of Cameroon. This region constitute with north region the sudano-sahalian zone of Cameroon. It is situated between 8º36'' to 12º54'' of the North latitude, and 12º30'' of the East longitude (IRAD, 2008). It covers a total area of 10.2 million hectares which 0.56 million are occupied by crops. The climate is characterized by monomodal rainfall type variable in intensity and in duration. The annual rainfall average is estimated around 750mm of water. It varies from 400mm to 1200mm a year from North to South. The average temperature can reach 27°C in Maroua while the maxima are from 40°C to 45°C in April (IRAD, 2008). The lowest temperatures are noticed between December and January. Two agro-climatic spaces are distinguishable: the flat of Mora, Maroua, Kaélé and Bec de Canard, where the rainfall risk are high and the piedmonts and mountains where the climatic risk are more limited because of the relative abundant rainfall (800mm to 900mm) and the good distribution of rainfall. The cultivated cereals in the region are: millet, sorghum, maize and rice. Peanuts and cowpea are the main produced leguminous crops. The main dominant cash crop is cottony.

Samples collection: Samples were collected in fifteen cowpea production basin of the far-north region, distributed in five divisions out of six that count this region. The reasonable method based on the importance of cowpea production, accessibility and the communication facility with the farmers was used for this stage. Collection was effectuated from September to November 2019 at the stage of preflowering, flowering and the pod formation. Interviews were conducted with some farmers in each village on the impact of the disease and on the orientation in the widest cowpea farm for sampling. Three to five farms per village were prospected and three to five plants with wilting symptoms caused by *Fusarium* per field were uploaded with their roots. They were brought to the laboratory in envelops. Before each sampling in farm, one to two symptomatic plants were longitudinally dissected and observed to be assured of discoloration of the vascular system due to *Fusarium spp*.

Isolation of *Fusarium spp* from cowpea: Isolation was carried out from the infected roots and stems tissues as described in the works of Amer *et al.* (2014). Roots were washed with the running tap water to remove the solids adhering particles and were dried under laboratory air. Roots and stems surfaces were sterilized in 95°C alcohol during five minutes. Each plant was then dissected longitudinally in two parts with sterilized scalpel following the median plan from root to stem. Two to three pieces of tissues showing chlorosis were cut and surface disinfected in sodium hypochloride 1% during 15 to 30 seconds (Pratt, 1982). They were washed trice in distilled water. Tissues pieces were dried under two filter papers and aseptically transferred on Potato Dextrose Agar (PDA) in the petri dishes of 90 mm diameter. Colonies developed after 2 to 4 days were purified by three successive subcultures.

Stimulation of sporulation: Four different culture media were used to identify the suitable culture for the production of spores: PDA 39g/l media, PDA 19,5g/l media, Malachite Green Agar (MGA) media and JOFF media. A small disc of the colony developed on PDA was cut with needle and aseptically transferred in each culture media mentioned above. The observations of spores were performed respectively after 5, 7 and 21 days of incubation. Slide was prepared by dropping one to two sterile distilled water on the blade. A little quantity of colonies was cut and putted on the blade and spread to favour the liberation of the eventual spores. The preparation was covered by lamella. Observations were done

under microscope to determine the presence or the absence of the different types of conidies.

Single spore culture

Spores suspension preparation: Spores suspension preparation was inspired on the method described by Niemeye and Andrade (2016). With forceps, a small quantity of homogenous colony developed on JOFF media was cut and introduced into 2ml of centrifugal tubes containing sterile distilled water. It was scratched to allow spores liberation. Spores were dispersed by Vortex for 30 seconds. Solution was filtered by Grid membrane of 0.45µm of pore to retain mycelium and to obtain the pure spores suspension.

Single spore culture technic: The single spore culture technic was for Niemeye and Andrade (2016). This technic was consisted of taking 3 to 5µl of spores' suspension with a micropipette and transfers it into each square drawn on the base of the petri dishes contemning Agar 2% media. Suspension was then spread in the square. Petri dishes were incubated under room temperature during 16h to 24h and then exanimated under stereo microscope. Each square was observed by dissected microscope and the single germinated spore in good position was removed and transferred on PDA. Three to four germinated spores were individually deposited on solid Potato Dextrose Agar (PDA). After 4 to 5 days of incubation, a unique colony was transferred on the new PDA culture media. After 5 to 7 days of incubation, strains were conserved under 4°C for next studies (Choi, 1999).

Characterization of *Fusarium spp*: Characterization of *Fusarium spp* is based on Booth (1977) and Summarell *et al*. (2006) works. The colonies discs of same dimension of *Fusarium spp* were taken and transferred aseptically on PDA media in petri dishes. Temperature, relative humidity and light were for room conditions. Observations were done from third day of incubation to 21days of incubation. Colonies aspects and their colours were noted. At the same time, colonies discs were putted on JOFF culture media for spores' stimulations. After seven days of incubation, a small quantity of colonies developed on Joff media was cut and deposited in 2ml of distilled water and then agitated with vortex to allow the liberation of spores. Two to three drops of spores' suspension were spread on the slide and covered with coverslide. Types of spores, their forms and the visual appreciation of their concentration were determined under microscope. The choice of samples for pathogenicity test was based on the types of spores, forms of spores and the concentration of spores.

Pathogenicity test: Pathogenicity test were conducted by using a susceptible variety IT99K-573-2-1 (Omoigui *et al.,* 2018) and the resistant variety CB46 (Pottorff *et al.*, 2014) as plants materials. Soils constituted of sands and loam in a proportion of 1:1 sterilized into autoclave at 120°C during one hour were poured into pots. Seeds were planted in plastic pots measuring 20.5cm of diameter 19.5 cm of depth. The experience was laid out in completely randomized design replicated three times. A root dip method as described by Pottorff *et al.* (2012) was used for inoculation. Seven days after sowing, when the firsts leaves were expanded, the young plant were gently uploaded. Roots were washed with tap water to remove the adhering particles. Roots extremities were trimmed with sterilized scissors and then soaked in *FOT* spores suspension with the concentration of 10^6spores per millimetre during 1h. Observation of plants reaction to *FOT* was effectuated seven weeks after inoculation. It was based on the discoloration of vascular system of the plant uploaded and bisected longitudinally. Disease severity was evaluated according to a scale of 0 to 5 (Omoigui *et al*., 2018). Severity percentage was estimated on the following scale: 0= healthy plant with no signs of disease, 1= approximately 10% of the plant showing symptoms of the disease, 2= approximately

25% of the plant showing symptoms of the disease, 3= approximately 50% of the plant showing symptoms of the disease, 4= approximately 75% of the plant showing symptoms of the disease, 5= approximately 100% of the plant showing symptoms of the disease.The strains with the highest degree of severity will be selected for varietal screening for the resistance against *FOT*.

Re-isolation of the pathogen: Pathogens were re-isolated from the plant presenting the symptoms of the disease to fulfil Koch postulate. The isolation technic was what has described previously. Results were confronted with the first isolation to confirm the causes of the symptoms observed on the field.

Statistical analysis: Variance analysis followed by the Tukey test to separate means, were carried out by R software.

RESULTS AND DISCUSSION

Isolation of the pathogen: Isolated *Fusarium spp* were obtained from cowpea plants presenting Fusarium wilt symptoms. The colonies were developed after 2 to 4 days of incubation on Potato Dextrose Agar media (PDA) from the roots and stems tissues with wilting symptoms. In total, 120 isolates were obtained at the beginning with a variety of colour (white, rose and white butter). These results are similar to what are described by Sumarel *et al*. (2006), Omoigui *et al*. (2018) who reported that *Fusarium oxysporum* colonies' forms and colours are varying on PDA. These colours are white, pink, violet or sometime dark depending on the strains.

***Fusarium spp* sporulation:** The observation of slide under microscope in the period from seven days after incubation to 21 days was revealed the absence of sporulation on PDA culture media for all isolates obtained from infected roots and stems tissues. According to Leticia *et al*. (2017) the richness of PDA media in nutrients elements and the great content of carbohydrate complex explain the inefficiency of that media to stimulate sporulation. In contrary, it favours the rapid growth of mycelium and inhibits sporulation. Thus, many previous searchers had used others cultures media to stimulate sporulation, and PDA media for colony characterization (Rejeki *et al.*, 2014; Chen and Chung, 2017). These results contradicting previous works carried out by Emberger and Nelson (1981), Hye *et al.* (2012) and Wamalwa *et al*. (2018**)** who showed the development of spores on PDA media under particular conditions like fluorescents light 12hour on/off cycle. As sporulation on PDA requires a well-equipped laboratory, to obtain conidia in low equipped laboratory, Sumarel *et al*. (2006) affirm that, others cultures media like Komada, Malachite Green Agar (MGA) and Corn Meal Agar (CMA) can stimulate the sporulation of *Fusarium oxysporum*. Sporulation was observed on MGA and Joff culture media for 80 isolates. Nevertheless, others isolates did not sporulate in any culture media. Different colours of the colonies developed from single spore on PDA were observed after seven days of incubation. Colours of superior side were generally different from the low side as presented on figure1.

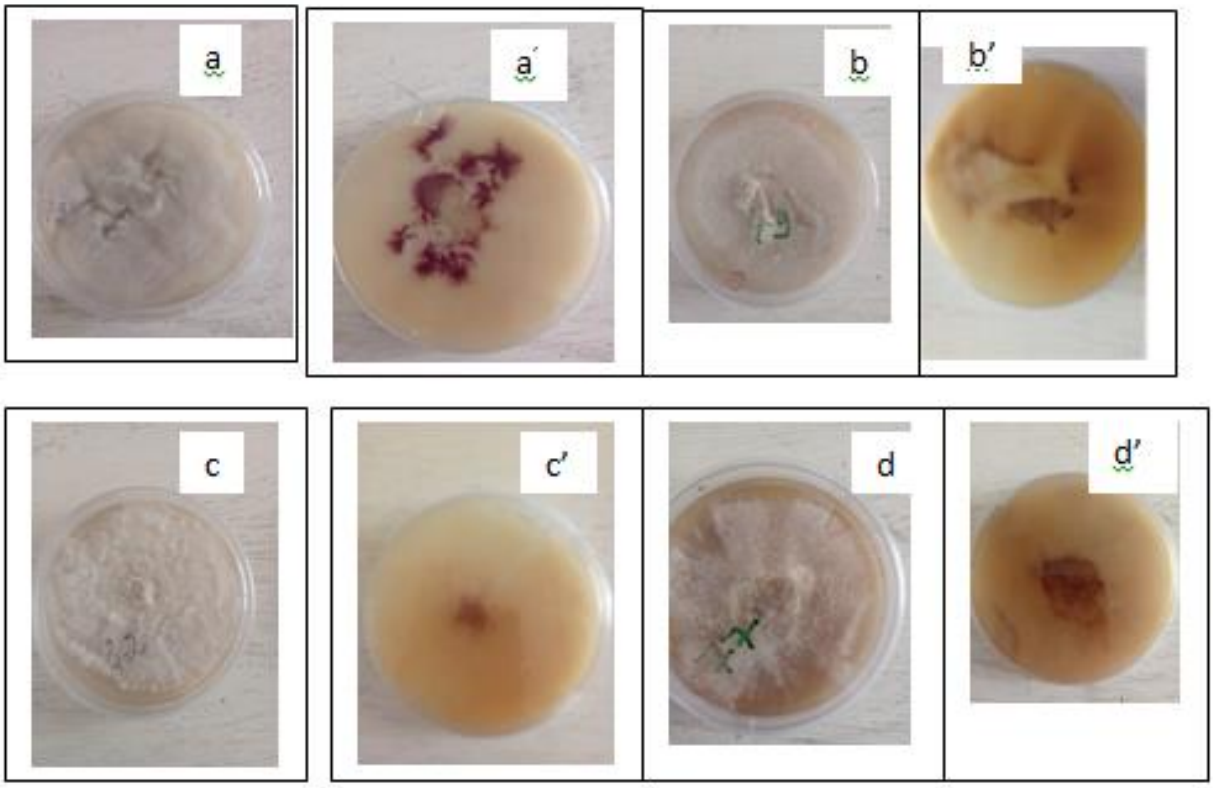

Figure 1: Colonies of *Fusarium oxysporum* on PDA. a, b, c and d = superior face. a', b', c', and d'=low face

The violet colour of the mycelium on PDA (Fig 1a') as specific character of *Fusarium oxysporum* (Sang-Do *et al.*, 2007**)** was observed on some isolates.

Macroconidia and microconidia: Conidial types observed were different depending on the isolates: macroconidies for some, microconidies for others and both for other isolates (Fig 2). Macroconidia were fusiform, arched with thin extremity or straight for others. Those macroconidia were all septed. Three to five septa per macroconidia were numbered but three septa were more represented. Those results are similar to what are described by Sumarel *et al.* (2006), Sang-Do *et al. (*2007) and Pirayes *et al*. (2018). Results show that microconidia are generally more numerous than macrocodia. They are not septed or have exclusively one septum per microconidia. They are elliptic, ovoid or reniforms. Those microcodies traits characterized *Fot* as reported by Antonia and Maria (2006).

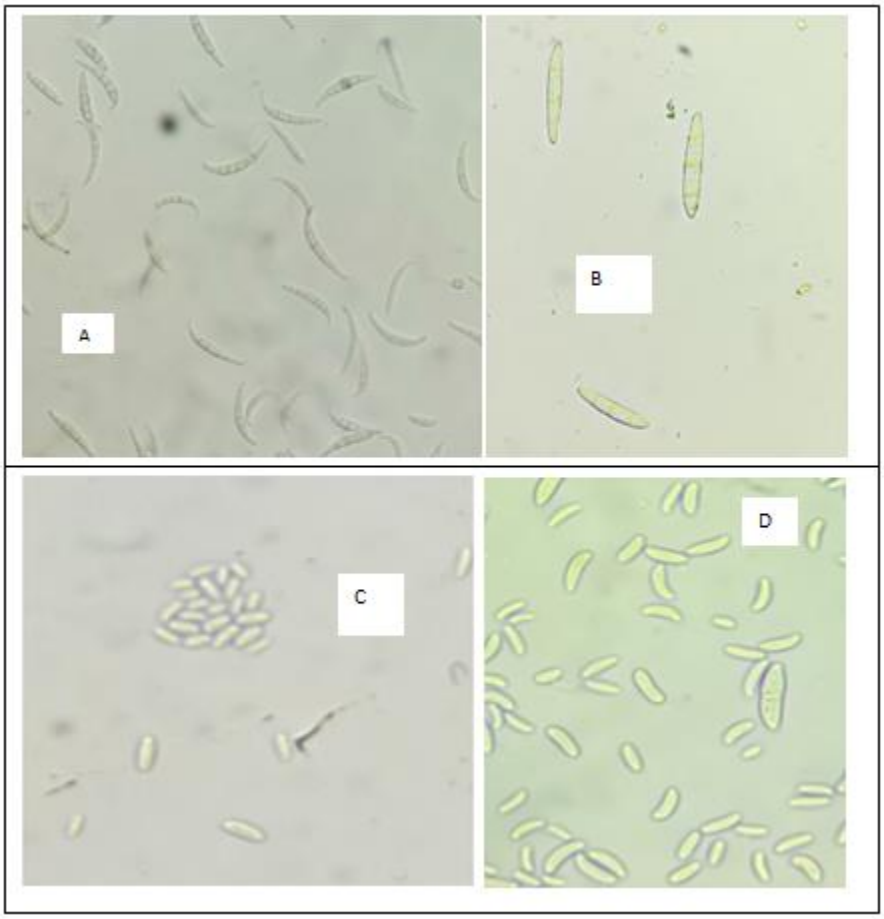

Figure2 : Conidies, A et B= macroconidies, C = microconidies developed in false head, D= microconidies

Chlamydospores: Results revealed the presence of monophialid and intercalary chlamydospores (Fig 3). Monophialid was short in all observed isolates. According to previous works carried out by Sang-Do et *al.* (2007) and Lombard *et al*. (2019), those characters are the distinctive traits that differentiate *Fusarium oxysporum* from others species of the same genre. This shown that the isolates belong to this specie. Visual appreciation of spores' concentration has revealed the variation in concentration from one isolate to another. Isolates choice for the pathogenicity test is based on the forms and types of conidia, the ability of strain to relatively produce a great number of spores and the types of spores.

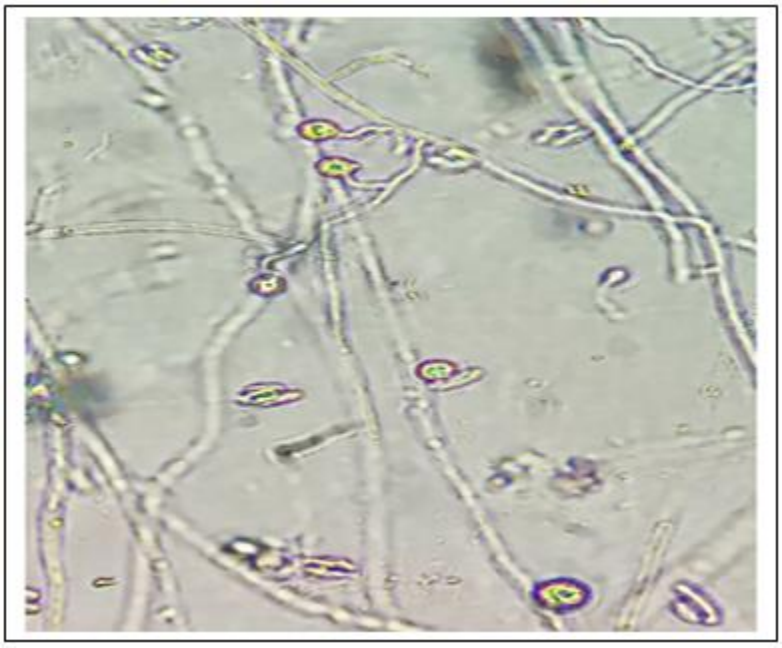

Figure 3: chlamydospores

Pathogenicity test: The species *Fusarium oxysporum* has several strains among which some are pathogens and others are saprophytes. Pirayes *et al*. (2018) reported that it is difficult to distinguish morphologically the pathogenic forms from the non-pathogenic forms. Pathogenicity test allows then to show the pathogenicity character of the isolates. Fusarium wilting symptoms (Fig4 a and b) were identical to the diseased plants samples collected on the field. Chlorosis was started on the old leaves and then followed latter by wilting. The vascular system of the wilted plant was brown. Nevertheless, the severities of the strains vary with the isolates. Thus, variance analysis shown significant difference ($P<0.05$) between isolates. No external visual symptom was observed on CB46 variety. Nevertheless, symptoms were observed in the vascular system after roots and stems are dissected. The effect of pathogens is limited compare to what is observed on IT99K-573-2-1. Those results may proof the absence of race 4 among tested isolates because CB46 was initially developed for the resistance against *Fot3* but it is very susceptible to *Fot* 4(Pottorff *et al.*, 2014).

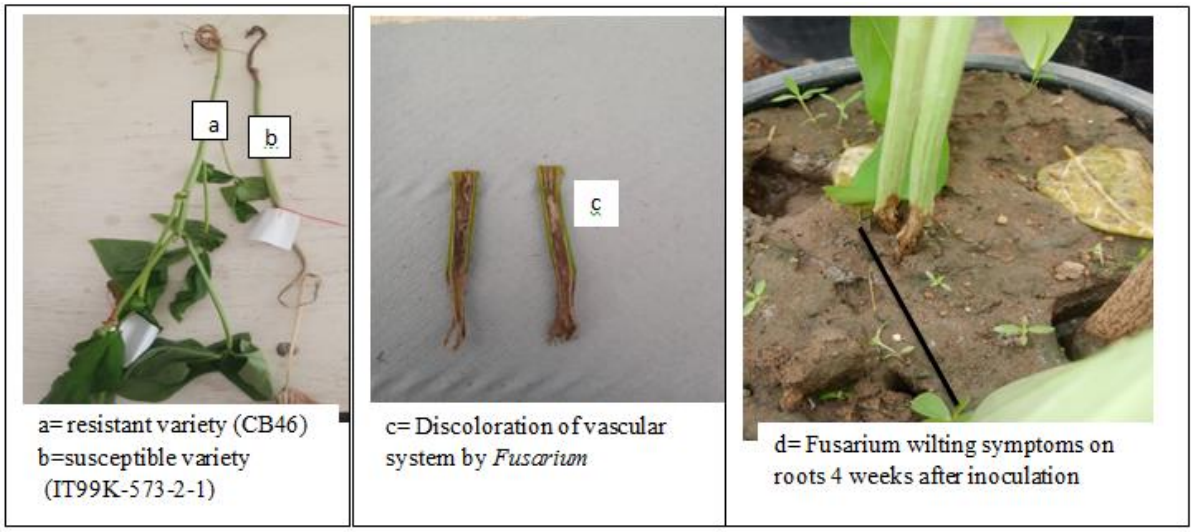

Figure 4: symptoms of Fusarium cowpea wilt disease

The results of table 1 show that, strain 7 follows with strain 6 are the more virulent. The first strain may be then considered as the more virulent in the far-north region among tested isolates. CB46 is identified as tolerant or resistant to all isolates. In the contrary, externals symptoms characterized by chlorosis, wilting and later the death of the plant were observed on IT99K-573-2-1 variety. These symptoms are similar to those caused by *Fusarim oxysporum sp tracheiphilum* on cowpea **(**Antonia and Maria, 2006). Therefore, isolates are identified as forma specialis of the species. The variety is confirmed as susceptible.

Tableau 1: severity of the isolates

Strains	**Severity**	
	IT99K-573-2-1	**CB46**
Ech7	4.00^{a}±1.00	2.33^{a}± 0.57
Ech6	3.00^{ab}±1.73	1.00^{ab}±1.00
Ech1	2.33^{abc}±1.00	0.66^{ab}± 0.57
Ech10	2.33^{abc}±1.00	0.33^{b}±0.57
Ech9	2.33^{abc}±1.52	0.66^{ab}±0.57
Ech8	2.33^{abc}±1.52	0.00^{b}±0.00
Ech2	2.33^{abc}± 0.57	0.00^{b}±0.00
Ech4	1.66^{bcd}±0.57	0.33^{b}±0.57
Ech5	1.33^{bcd}± 0.57	0.33^{b}±0.57
Ech3	1.00^{cd}±1.00	1.00^{ab}± 1.00
Testimony	0.00^{d}±0.00	0.00^{b}±0.00

The re-isolated strains from diseased plant were morphologically identical to those which are obtained from the diseased plant collected directly on the farm. This revealed that isolated strains are responsible of cowpea wilting in the Far-North region of Cameroon.

CONCLUSION AND APPLICATION OF RESULTS

Fusarium cowpea wilting caused by *Fusarium oxysporum s.f tracheiphilum* is widely distributed in the Far-North region of Cameroon in the sense that symptoms were observed almost in all prospected cowpea farms. Strains responsible of the disease were collected and isolated according to the methodology described in previous works. These pathogens have variable degree of virulence. Thus, strain 7 was identified as the more virulent in the current study. It is considered as responsible of damages caused by *FOT* in far-north region. This strain can be used for varietal screening for the resistance against the pathogen in the perspective of plant breeding program.

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