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EFFICACY OF CYMBOPOGON CITRATUS (DC) STAPF AND AZARIRACTHA INDICA L PLANT EXTRACTS IN VITRO AND IN THE CONTROL OF ARTIFICIAL INFESTED SOIL BY RALSTONIA SOLANACEARUM IN WEST BURKINA FASO

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Abstract:-

Ralstonia solanacearum is one of the soil-borne pathogens that affect tomato (Solanum Lycopersicum L.) production. In response to its damage, farmers mainly use synthetic chemicals products that are harmful to the environment, the users and the consumers. In order to reduce chemical product use, the alternative control methods based on plant extracts are developed to control R. solanacearum on tomatoes. The objective of this study is to build effective control methods against R. solanacearum the agent of tomatoes wilt. For that, we first tested the efficacy of Cymbopogon citratus and Azadirachta indica extracts on the growth of Ralstonia solanacearum in vitro using the method base on poisoned media. Our second activity was to disinfest soil contaminated by R. solanacearum in green house by incorporating some quantity of citronella or neem almond in soil infested by R solanacearum. On poisoned media, we shown that both plants have reducing effect on the development of R solanacearum when compared with the control. The incorporation of C. citratus (15%, 20% and 30%) and A. indica (4%, 5%, 6%, 7%, 8% and 10%) powders inhibited the survival of the bacterium in the artificially infested substrate 70 days after treatment. This study confirms the hypothesis that plant extracts of C. citratus and A. indica can be used as an effective method to control Ralstonia solanacearum on tomatoes.

Key words:-*Solanum Lycopersicum, Ralstonia solanacearum, soil disinfestation, plant extracts, Burkina Faso*.

1. INTRODUCTION

The tomato (*Solanum lycopersicum* L.) is the most consumed vegetable after potato in the world. Its production increased from 141,648,137 tonnes in 2008 to 182,256,458 tonnes in 2018, an increase of more than 20% in ten (10) years, making the tomato the most producing vegetable, ahead of watermelon and cabbage (Faostat, 2018). In Burkina Faso, it is the second most important garden product as much as production area than production after onion. Its production is done by many farmers during the dry season and generates a lot of income for producers and distributors (Son, 2018). Indeed, the total sales value of tomato in 2018 was estimated at more than 78 million CFA, this represented 20% of the market gardening turnover. It therefore plays a very important socio-economic role for the country. Unfortunately, this speculation encounters several biotic and abiotic constraints that limit its production. The tomato is infected at all stages of its development by bio-aggressors, especially soil borne pathogen are very damaging in field (Soro *et al.*, 2011). Among them, *Ralstonia solanacearum*, the agent of bacteria wilt, is one of the best known and the most studied in the world. The bacterium can cause yield losses up to 90% in tomato crops (Cariglia, 2007). Despite numerous studies undertaken on a long time, bacteria wilt remains a very damaging disease of which control method are not still very efficient. The intensification of agriculture has largely contributed to increasing the pressure of these pathogens. Nowadays with the policy of reducing chemical product in plant protection, soil borne pathogen, particularly *Ralstonia solanacearum*, is became worrying for gardener. Fortunately, many results shown that plant extracts have anti-microbial properties (Hien, 2017; Djiguemdé, 2018; Ouédraogo, 2019). The studies in this domain can be improved in order to control *R. solanacearum* on tomato crops in Burkina Faso. This study was initiated to experiment the efficacy of *Cymbopogon citratus* and *Azadirachta indica* on *R. solanacearum* the tomato bacteria wilt. The overall objective is to contribute to the development of effective control methods against *R. salanacearum*.

2. Material

Plants species tested

The plants species selected for this test were *Cymbopogon citratus* and *Azadirachta indica*, which have proved their effectiveness seed and soil treatment against *Fusarium oxysporum* (Hien, 2017).

-Azadirachta indica

(A.) Juss: is a tree of the Meliaceae family, native to eastern India. The tree can reach 20 m in height and 2.5 m in circumference and has been planted in Africa and cultivated in all tropical regions for various uses, including medical purposes (the leaves are traditionally used in the treatment of malaria, oedema and rheumatism). It's contained some Chemical substances like sterols, terpenes, flavonoids. Bonzi *et al*. (2012) prove its efficacy in sorghum seed treatment against *Phoma sorghina.*

Cymbopogon citratus (D.C.) Stapf: also called "citronella" is an aromatic inveterate grass of the family of *Poaceae* cultivate for its medicinal properties and essential oils use in cosmetics and perfumery (Okezie and Agyakwa, 1987). Bonzi *et al*. (2012) also shown his efficiency in sorghum seed treatment against *Colletotrichum graminicola* and *Phoma sorghina*.

Ralstonia solanacearum

We have tested the extracts of these plants against *Ralstonia solanacearum* which is a soil borne bacterium. The bacterial strain used is a local NMDG 111 strain of Phylotype I-31 with 100% aggressiveness (Ouédraogo, 2019).

Chemical fungicides

Fungicides such as Bacitracin (Sigma B-0125), Chloramphenicol (sigma C- 3175), PenicillinG (sigma P-3032), Ortiva and TTC (Triphenyl tetrazolium chloride) have been used to inhibit the growth of fungi saprophytes in culture media.

3. Methods

3.1 Effect of plant aqueous extract on the development of *R. solanacearum*

31.1. Reproduction of *Ralstonia solanacearum* strain on LPGA

The LPGA medium was prepared by mixing 7 g Yeast, 7 g Peptone, 7 g Glucose and 15 g Agar in 1000 ml of distilled water. The mixture was sterilised at 120°C for 20 min. After cooling, it is dispatched in Petri dishes under aseptic conditions. Under aseptic conditions, a sample of the bacterial strain was spread on medium and incubated in sweeting-room for 24 hours.

3.1.2. Inoculum preparation

A 10^8 CFU bacteria suspension was reproduce by collecting the developed colonies in a sterile Ependoff tube containing 1 ml of sterile distilled water. This mixture is then vortexed to homogenise the suspension. The spectrophotometer was used to determine the Optical Density (OD) of the suspension which we diluted to obtain a final suspension with an OD of 0.15.

3.1.3. Preparation of aqueous extracts and culture media

The shade-dried *Cymbopogon citratus* leaves were cut into small pieces and then ground into powder using a grinder. Thirty (30) grams of lemongrass powder were macerated in 100 ml for 24 hours at room temperature in the laboratory. At the end of maceration, the extract is obtained by pressing and then filtering through a fine cloth.

For *Azadirachta indica* the almond powder at 70, 80, 100 g were macerating respectively in 1000 ml of sterile water at 25 °C for 24 hours. At the end of this period, the extracts are obtained by pressing and filtering through a fine cloth giving respective concentrations of 7, 8 and 10%.
The SMSA poisoned medium was obtained by mixing 1 g Casein, 5 ml Glycerin, 5 g Peptone: 9 g Agar in 1000 ml of extract. The mixture has been sterilised at 120°C. After cooling, it was divided in Petri dishes under aseptic conditions.

3.1.4 Inoculation of culture media and incubation
The inoculation of bacteria suspension on the culture media was done according to the method of nutrient substrate poisoning. 15 µl of the bacteria suspension. This suspension is deposited directly into the poisoned culture medium and then spread evenly over the entire surface using a sterile handful.

3.1.5. Experimental device
Each plant extract was tested on the bacteria in a completely randomized block device with seven (7) treatments and four (04) replicates per treatment. Thus, the treatments carried out are as follows:
TE: SMSA water control medium;
EAi 7%: SMSA medium with 7% neem seed aqueous extract;
EAi 8%: SMSA medium with 8% neem seed aqueous extract;
EAi 10%: SMSA medium with 10% neem seed aqueous extract;
ECc 15%: SMSA medium with 15% citronella water extract;
ECc 20%: SMSA medium with 20% citronella water extract;
ECc 30%: SMSA medium with 30% citronella water extract.

3.1.6. Collect and analysis data
To measure efficacy of plant aqueous extract on the development of *R. solanacearum*, 10 ml of water were added on the surface of each colony cultivate in the Petri dish. Using a sterile anse, we made rotating movements to disperse bacteria in distilled water. The suspension was collected from each Petri dish in order to measure the optical density compared to the control.
The data collected were analysed using IBM SPSS Statistics version 22 software. The averages were compared using the Student Newman and Keuls multiple classification test at 5% threshold. The results are presented in table

3.2. Efficacy of plant extract in the treatment of infested soil by *R. solanacearum*
3.2.1. Preparation of treatments based on plant extracts
For this test, the treatments based on plant extracts have a powdery formulation and have been prepared as follows:

- For lemongrass: The freshly harvested lemongrass leaves are first dried in the shade and then cut into small pieces before being ground into powder using a grinder.
- For *A. indica*: Seeds have first been well dried and then the almonds were crushed to obtain the powder.

3.2.2 Artificial infestation and treatment of infested substrate by plant extracts
At this stage of the experiment, potting soil was previously steam-sterilised and each pot was given 2.5 litres of substrate. Twenty-five (25) ml of bacteria inoculum concentrated at 10^8 CFU was then brought into the pots. One week after this inoculation of the substrate, we proceeded on treatments by plant extracts. Thus, a quantity of plant powder for each treatment was measured according to the volume of soil. Before their application, we have deducted the quantity of substrate corresponding to those of plant powder before add it's in pot. After the application of the different treatments, the pots were watered and then the substrate was regularly turned to ensure a good mixture of soil and plant extracts.

3.2.3. Experimental device
A completely randomized block experiment design with four (04) replicates for each treatment was used. Treatments based on extracts of *A. indica* and *C. citratus* that showed efficacy on the growth of the bacterium *in vitro* were tested in green house. The treatments were as follows:
TE: substrate without any treatment
EAi 4%: substrate mix with *A. indica* almond powder at 4%
EAi 5%: substrate mix with *A. indica* almond powder at 5%
EAi 6%: substrate mix with *A. indica* almond powder at 6%
EAi 7%: mix with *A. indica* almond powder at 7%
EAi 8%: mix with *A. indica* almond powder at 8%
EAi 10%: mix with *A. indica* almond powder at 10%
ECc 15%: substrate mix with citronella powder at 15%;
ECc 20%: substrate mix with citronella powder at 20%;
ECc 30%: substrate mix with citronella powder at 30%.

3.2.4. Isolation and characterization of bacteria from soil samples
For the detection of bacteria in treated soil, 300 g of soil were sampling in each pot at 70 days after artificial inoculation. In the laboratory, 10 g of soil was taken from each soil sample (300 g) and mixed with 50 mL of extraction buffer (0.85% NaCl) and stirred at 250 rpm for two hours. The homogeneous mixture was left to stand for at least 30 minutes to obtain

a suspension. From each suspension, 1 mL (of the floating part) was pipetted to make eight dilution runs at decreasing concentrations (10) into eight vials containing 9 mL of sterile distilled water. The LPGA medium was used for detection of *R. solanacearum*. Ten (10) µL of each dilution were pipetted and spread by Petri dishes eight Petri dishes were inoculate by concentration. A reference strain of *R. solanacearum* bacteria was also spread as a control. After inoculation, the Petri dishes were incubated for 72 hours at 28-30°C. At the end of incubation period, we proceeded to morphological identification of colony.

The pure colonies suspected were transfer on LPGA medium and incubate for 48 hours. These pure bacteria colonies were used to prepare bacteria suspensions at 10^8 CFU mL-1 which were used for the identification of *R. solanacearum* Phylotypes by Multiplex PCR (Fegan and Prior, 2005). The PCR involved four (4) specific phylotype forward primers and one (1) reverse primer common (primer acronym) to all Phylotypes and primers 759 and 760 to amplify the specific 280 bp band of *R. solanacearum*. It was performed with a reaction volume of 25 µL.

This volume is composed of 14.5 µL of sterile distilled water, 5 µL of MultiPlex Mix, 0.5 µL of each of the 7 primers and 2 µL of bacterial suspension at 10^8 CFU.mL-1. The samples undergo an initial denaturation phase of 5 minutes at 96°C in a thermal cycler, followed by 30 reaction cycles: (i) denaturation for 1 min at 94°C, (ii) hybridization for 90 seconds at 59°C, (iii) elongation for 90 seconds at 72°C, followed by a final elongation phase of 20 min at 72°C. The PCR amplifications were performed in a thermal cycler. The PCR products were then separated by electrophoresis on 1% agarose gel. The revelation is made thanks to the red gel which is brought to the agarose gel before it is poured on a U-shaped plate. Visualization is carried out on a UV table.

4. Results

4.1. Effect of plant extracts on the growth of *Ralstonia solanacearum*

Plant aqueous extract significatively reduce the growth of *R. solanacearum* on poison media compare to the control (Table I). All the concentrations of citronella, EC 15%, 20% and 30%, as well as neem extract at 10%, were more effective in reducing *R solanacearum* development compared to the control. There was no significant difference between optical density of citronella and neem extract at 10%. With *A indica* aqueous extract, our results shown that the efficiency of extract were increasing with the concentration of this extract in the medium. This result shows that plant extract of citronella and *A indica* in mixture with some substrate was not so favourable for the development of *R. solanacearum*.

Table I: Effects of plant extracts on the growth of *Ralstonia solanacearum*.

Traitements	Densité optique Sur milieu empoisonné
TE	2.00d
ECc 15%	0.11a
ECc 20%	0.11a
ECc 30%	0.14a
EAi 7%	0.46c
EAi 8%	0.22b
EAi 10%	0.05a
Valeur de F	**679.893**
Probabilité	**0.000**
Signification	**HS**

In the same column, the means affected by the same letter are not significantly different at the 5% threshold (Newman Student Keuls test); TE: water control; EC: citronella water extract; EN: neem oilcake water extract; HS: highly significant.

4.2. Efficacy of plant extract in the control of *R. solanacearum* infested soil by

After isolation on LPGA and characterization of suspicious colonies by PCR, the result shown that *R. solanacearum* was not present in soil artificially infested and treated by plant extract of *C. citratus* or *A. indica*.

Table II: Detection of bacteria in treated soil

Treatment	Presence/absence of bacteria
TE	+
EAi 4%	-
EAi 5%	-
EAi 6%	-
EAi 7%	-
EAi 8%	-
EAi 10%	-
ECc 15%	-
ECc 20%	-
ECc 30%	-

Sign (-) : absence of Ralstonia solanacearum ; Sign (+) : présence of Ralstonia solanacearum , EAi : Extract of Azadirachta indica, ECc : Extract of Cymbopogon citratus

5. Discussion

Effect of plant extract on the development of *R. solanacearum*

In Africa, many plants are known and used for their antimicrobial activities (toxic, repellent, anti-appetence) against a wide range of bio-aggressors. The use of pesticidal plants can be a promising alternative for the management of bio-aggressors in vegetable crops (Yarou *et al*., 2017).

Measuring effect of aqueous extracts of *Cymbopogon citratus* and *Azadirachta indica* on *Ralstonia solanacearum* shows that these plants extract negatively affect the development of this bacterium. On media with aqueous extracts (poisonous medium), citronella extracts showed more efficiency in reducing the development of *R. solanacearum* compared to the control. Similar results were obtained by Deberdt *et al.* (2012) who have shown that aqueous extract of *Allium fistulosum* at 50 and 100% inhibited the growth of *Ralstonia solanacearum in vitro*, and that its use in treatment of soil infested by *R. solanacearum*. The efficacy of *Ocimum gratissimum* extracts also has been proved by several authors such as Adiguzel *et al* (2005), Wagura *et al* (2011), Owoseni and Sangoyomi (2013), Kumar *et al* (2017), Yarou *et al* (2017) on tomato culture.

In the same order, our results were indicated the efficacy of the aqueous extract of *Azadirachta indica* almond at 7, 8 and 10% on the development of *R. solanacearum*. Several organs of this plant have been proved to be effective against this bacterium. Sukanya *et al.* (2009) and Narasimha *et al.* (2015) shown the anti-bacteria activity of methanol extracts of *A. indica* leaves *against R. solanacearum*. Narasimha *et al*. (2012) in testing various plant extracts *in India* indicated that *A. indica* and *Punica grenatum* have shown a strongest inhibitory activity on *R. solanacearum* but, those of guava, papaya and turmeric were not so efficient. To resolve the problem of soil infestation by *R. solanacearum*, plant aqueous extract of citronella and *A. indica* almond can be experimented as an alternative solution according to their efficiency *in vitro*.

Efficacy of plant extract in the control of *R. solanacearum* in infested soil

This study focused in particular on the analysis of the sanitising effect of the extracts of these two plants on substrate already infested by the pathogen. Experience shows that both plants inhibit the survival of the bacterium because the bacterium could not be detected in the substrate after treatment. Among the re-isolated bacteria, those that had a morphological resemblance (milky white appearance in the LPGA medium) to *R. solanacearum* were discriminated by PCR. The absence of the bacterium in the substrate could be explained by antibacterial properties contained in plant used which, when incorporated into the infested substrate, diffused their toxic substances against pathogens, (including the bacterium responsible for tomato wilt). Some plant organs when dried, and turn into powders, can be used not only as organic matter to amend soil but can also mix with soil for their antimicrobial substances that protect host plants against pathogens. Paret *et al* (2010) showed that *R. solanacearum* would not be detected in pots infested and then treated with *Cymbopogon martini* and *C. citratus* essential oils at 0.07 and 0.14% and the treatments did not induce a reduction in ginger yield. The work of Narasimha *et al* (2015) revealed that *A. indica* leaf extracts have an inhibitory effect against *R. solanacearum* compared to antibiotics. Pontes *et al*. (2011) tested the effect of incorporating *A. indica* leaves into infested soil to control bacterial wilt and they obtained the suppression of wilt symptoms on plants.

6. Conclusion

At the end of this study, we can notice that *Cymbopogon citratus* (15-30%) and *Azadirachta indica* almond (10%) aqueous extracts significantly limited the development of *Ralstonia solanacearum in vitro*.

The Incorporation of *Azadirachta indica* almond powder (4% to 10%) and *Cymbopogon citratus* leaf powder (15% to 30%) efficiently control *Ralstonia solanacearum* in artificial contaminated substrate.

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