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RESEARCH ARTICLE

BACTERIAL WILT OF TOMATO IN CENTRAL CÔTE D'IVOIRE: IDENTIFICATION OF THE CAUSAL AGENT AND CONTROL BY EXTRACTS OF ALLIUM FISTULOSUM AND HYDROCOTYLE BONARIENSIS.

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Abstract

Tomato is one of the major new sources of income for many farmers in central Côte dôIvoire. However Plant wilting undermines tomato cultivation in Central Côte dôIvoire. The purpose of this paper is to help improve the production of that vegetable through both identification and non-chemically control of the causal agent of the bacterial wilt. During the phytosanitary survey, the disease prevalence and severity were determined in five tomato plantations in Yamoussoukro. The pathogen associated to bacterial was isolated and tested for pathogenicity. The sensitivity of the causal agent to the ethanolic extracts of *Allium fistulosum* and *Hydrocotyle bonariensis* was then evaluated using the well method and dissemination on SPA medium; subsequently applied on artificially infected plants. Several symptoms including leaf flaccidity, chlorosis, browning between veins, leaf drying and browning of the vascular system were observed. Prevalences ranged from 30 to 85% while severities ranged from 2.3 to 4.63. The bacteria *Ralstonia solanacearum* responsible for the disease showed high sensitivity to the ethanolic extract of *A. fistulosum* and a moderate sensitivity to *H. bonariensis*. On inoculated tomato plants, extracts of *H. bonariensis* and *A. fistulosum* brought down the prevalence of wilting by 31.67% and 35%, respectively. Control trials could be conducted in the field to determine the optimal doses of extracts that can substitute synthetic pesticides proven very expensive and relatively toxic for consumers.

Introduction:

Tomato (*Lycopersicon esculentum* Mill.) is an important vegetable crop whose fruit is much consumed across the world. It is a vegetable rich in proteins, vitamins, minerals, secondary metabolites, dietary fiber (Shankara *et al*., 2005). Tomato is also an important source of income for smallholders (Popoola *et al*., 2015). In Côte d'Ivoire, its estimated production of 32 364 tons per year (Popoola *et al*., 2015). (FAO, 2011) is well below the about 100 000 tons of food requirements of the populations (Soro *et al*., 2007).

Such low production is partly due both to a lack of crop promotion and to pathogens including bacteria, which represent nowadays the main limiting factors. Bacterial disease, in this case bacterial wilt, remains the major phytosanitary problem associated with that crop

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(Denny, 2006 ; Fondio *et al*., 2010). It begins with flaccidity and curving of the youngest plant leaves downward (epinasty) and the wilting of one side of the plant (Poussier, 2000). Yellowing partially followed by the drying will happen until the death of the entire plant (Digat and Caffier, 1996). Tomato wilt occurs in all tropical, subtropical and temperate zones of the world (Timatin *et al*., 2016). It limits the production of several Solanaceae such as tomato, eggplant, tobacco, pepper and other important crops such as groundnuts and bananas (Popoola *et al*., 2012). About 450 plant species are reported to be the hosts of the pathogen (Nguyen and Ranamukhaarachchi, 2010). In Côte d'Ivoire, a study conducted by Fondio *et al*. (2013), showed that in the South, the incidence of bacterial wilt ranged from 28.6 to 57.2%, resulting in significant yield losses. Severe wilting has recently been observed in several plantations in central Côte d'Ivoire-the new producing zone of the commodity. However, it is challenging to control the disease due to the high variability of the pathogen, its ability to survive in a various environments and its wide range of host plants (Teng *et al*., 2010). The use of plant extracts with biocidal properties and broad spectrum of action could be considered. Indeed, (Hassan *et al*., 2009) showed that several aqueous plant extracts would protect potato plants against bacterial wilt in greenhouses (under cover) and in the open field (outdoors). Allicin, present in the aqueous extract of *Allium sativum*, demonstrates antibiotic activity against a wide range of phytopathogenic bacteria and fungi (Slusarenko *et al*., 2008; Lebeau *et al*., 2011; Abo-Elyours *et al*., 2012; Péninna *et al*., 2012). Therefore, the use of plants with antimicrobial properties proves effective in controlling the disease. This paper aims to help improve tomato production by non-chemical control of bacterial wilt.

Materials And Methods:

Materials:

The plant material used consisted of symptomatic tomato plants, apparently healthy 21-day tomato seedlings (stage 5 of the leaf), then *Allium fistulosum* and *Hydrocotyle bonariensis* leaves. Symptomatic samples of tomato plants were collected at Yamoussoukro in central Côte d'Ivoire (Latitude 6° 48 North and Longitude 5° 30 West). They were used to isolate the pathogen. The apparently healthy tomato seedlings evaluated not only the pathogenicity of the isolated strains but also the *in vivo* sensitivity of the pathogenic strain to plant extracts. These extracts were prepared from leaves of *Allium fistulosum* and *Hydrocotyle bonariensis* respectively purchased commercially and collected on the experimental site of the University Nangui Abrogoua (Figure 1).

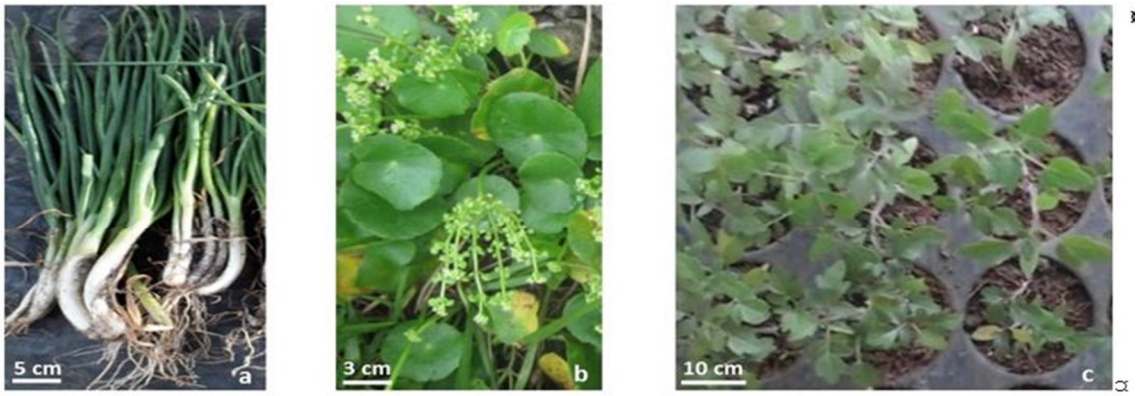


Figure 1: Plants used.

a: *Allium fistulosum*, **b**: *Hydrocotyle bonariensis* and **c**: Tomato seedlings

Sample Collection:-

Collection of symptomatic plants was carried out in five two-month-old tomato plantations of about 0.25 ha each and at least 15 km apart. The collection was made according to a pattern following a letter "X". Tomato plants with wilting were observed, described and photographed. Samples consisting of main roots (10) and stem segments (10) between the roots and the neck were then collected by plantation. The samples were kept in plastic bags and subsequently sent to the laboratory for pathogen isolation.

Determination of disease status in plantation:-

Assessing the prevalence of plant wilting:-

The prevalence of plant wilt in each plantation was also assessing using the same "X" pattern. Sixty (60) tomato plants were selected by plantation along both diagonals, on the basis of 30 plants per diagonal. The number of plants showing symptoms in relation to the total number of plants visited, representing the prevalence, was calculated according to the following formula:

$$PM\ (\%) = \frac{Pt}{N} \times 100$$

PM: Mean disease prevalence Pt = Number of plants showing symptoms
N = total number of plants selected.

Assessment of the severity of plant wilting:-
The wilting severity was assessed on the tomato plants visited. Only plants with symptoms were considered. The severity assessment was conducted according to the scale of (Mitsuro et al., 2013) ranging from 0 to 5:

0: no symptoms; **1**: partial wilting of a lower leaf; **2**: wilting of 2 to 3 leaves; **3**: wilting of all but from the first to the third leaf; **4**: wilting of all the leaves; **5**: death of the plant.

Score ranging from 0 to 5 was assigned to each diseased plant, depending on the intensity of the symptoms. The severity of wilting symptoms was calculated according to formula (Mitsuro *et al*., 2013):

$$S = \frac{\sum(P \times Q)}{N}$$

S: Disease severity; **P**: Disease score; Q: Number of plants with the P score; **N**: Total number of diseased plants

Identification of the causal agent for plant wilt:-
Bacterial oozing test or water glass test:-
A stem sample previously collected was placed in a test tube containing 250 ml of water. After three minutes, it was observed for the detection of milky white macerate in the test tube, characteristic of the bacterium *Ralstonia solanacearum*

Isolation of the bacteria associated with symptoms:-
Infected tomato stems were cut into small fragments (explants) of 10 mm. The explants were disinfected with 8°C sodium hypochlorite solution AT 3% for three minutes and then rinsed three times successively with sterile distilled water. After removal of the excess water on sterile absorbent paper, the explants were seeded on sucrose-petrone (SPA) agar medium at the rate of three explants per Petri dish. The cultures were then incubated in the laboratory for two days at room temperature of 25 ± 2 ° C.

The bacterial colonies developed around the explants were collected separately and then transplanted into new SPA media in order to obtain pure individual colonies. Using a platinum loop, the individual colonies were collected and seeded by fine striations.

Assessing pathogenicity of isolated bacteria:-
100 εl of bacterial inoculum at 2.7 10^7 UFC/ml concentration was used. The bacterial inoculum was soaked in sterile hydrophilic cotton and placed at the neck of the tomato plant. A paper tape was used to hold the cotton at the collar. Thirty tomato plants were inoculated with the bacterial suspension and 30 uninoculated plants served as controls. The experiment was repeated three times. The plants were placed under a closed shelter without contact with the soil. They were subjected to room temperature (25 °C) and daylight. Four weeks after inoculation, the symptoms appeared and were observed and described. In order to verify the involvement of the bacterial colony in wilt induction, a new bacterial oozing test was carried out and the bacterium was re-isolated on new SPA medium.

Assessing the antibacterial properties of plant extracts:-
Preparation of ethanolic plant extracts:-
The leaves of *A. fistulosum* and *H. bonariensis* were first washed three successive times with tap water and then placed on absorbent paper to dry the wash water. They were then dried at 20 ± 2 ° C for eight weeks. Then, the leaves were separately reduced to powder using a blinder. Ten grams (10 g) of each powder were dissolved in 500 mL of ethanol and macerated for 24 hours in the dark and at laboratory temperature (25 ± 2°C). The solution obtained was sterilized by filtration on sterile hydrophilic cotton according to the method of (Yao *et al*., 2017). Each raw extract 20 mg / mL concentration was diluted to 75 and 50%. The 15 mg / mL and 10 mg / mL concentrations were finally used for the *in vitro* test.

In vitro assessment of the antibacterial activity of extracts:
The SPA-medium well and dissemination method of (Khebichat, 2014) was used to assess the antibacterial activity of ethanolic extracts of *A. fistulosum* and *H. bonariensis*, *in vitro*. For this purpose, 100 εL of *Ralstonia solanacearum* suspension at $2.7.10^7$ CFU / mL concentration were spread on the SPA medium (sucrose-petion agar) contained in the petri dishes. Four 6-mm wells in diameter were then dug in the culture medium on which the bacteria were spread. The wells have been arranged diametrically opposed. For each Petri dish, 50 εL of each concentration of extracts were separately deposited in two wells and 50 εL of ethanol in the other two control wells. The Petri dishes were then sealed and incubated at 25 ± 2 °C. Seven days after incubation, the inhibition diameter of bacterial growth caused by the ethanolic extracts of the plants was measured using a graduated ruler. From these inhibition diameters, the sensitivity of the bacteria to the extracts was determined according to the scale of (Ponce *et al*., 2003): **Resistant (-)**: diameter less than 8 mm; **Sensitive (+)**; diameter between 9 and 14 mm; **Very sensitive (++)** diameter between 15 and 19 mm; **Extremely sensitive (+++)**: diameter higher than 20 mm.

In vivo assessment of the antibacterial activity of extracts:
The concentrations of ethanolic extracts of *H. bonariensis* and *A. fistulosum* that showed strong *in vitro* antibacterial properties were used for *in vivo* tests. Thus, 21-day-old tomato seedlings free from wilting were first transplanted into pots of 9.5 cm high and 13.5 cm thick containing previously sterilized forest soil. Seven days later, these seedlings were inoculated with 100 εL of bacterial suspension (2.7×10^7 CFU /ml) contained in cotton at the collar of each seedling. To assess the effect of extracts on *R. solanacearum* and therefore the development of plantlets, the following treatments were conducted:
T_0: Seedlings that have not been inoculated and have not received extracts (healthy control);
T_1: Seedlings having been inoculated and not receiving extracts (inoculated control);
T_2: Seedlings having been inoculated and having received the *A. fistulosum* extract;
T_3: Seedlings having been inoculated and having received *H. bonariensis* extract;

Each treatment was performed six times during 60 days (duration of the experiment), at a frequency of ten days. Ten (10) seedlings were used per treatment and the experiment was repeated 3 times. The experiment was conducted under a closed shelter. Sixty days after inoculation, the symptoms appeared and the disease prevalence and severity were determined.

Determination of the prevalence and severity of wilting in infected seedlings:
Wilting prevalence and severity of inoculated plants were determined for each treatment respectively according to previous formulas 1 and 2.

Statistical analyzes:
The Statistica 7.1 software was used to compare the mean prevalence and severity of tomato plant wilt inoculated with inoculated plantlets and the mean inhibition diameters of the bacterium by plant extracts. In case of significant difference, the Fisher LSD test was used to determine the homogeneous groups at Ŭ(5%) threshold.

Results and Discussion:

Results:

Symptoms observed at farm level:
Five different symptoms of bacterial wilt were observed on tomato plants in the five plantations visited. These include leaf flaccidity, chlorosis, browning between leaf veins, leaf drying and browning of the vascular system (Figure 2).

1. Flaccidity of leaves is characterized by the rapid wilting of young leaves at the hottest times of the day. Wilting is often reversible overnight at first and subsequently turns permanent (Figure 2A) ;
2. Chlorosis show by the yellowing of the lowest leaves (Figure 2B) ;
3. Browning between the veins shows as brown spots on the leaflets (Figure 2C) ;
4. Drying of the leaves is characterized by leaf burning (Figure 2D) ;
5. The Browning of the vascular system results in the necrosis of the conductive tissues of the stem showing as brown color (Figure 2E).

Figure 2: Symptoms of wilting observed on tomato plants in central Côte d'Ivoire

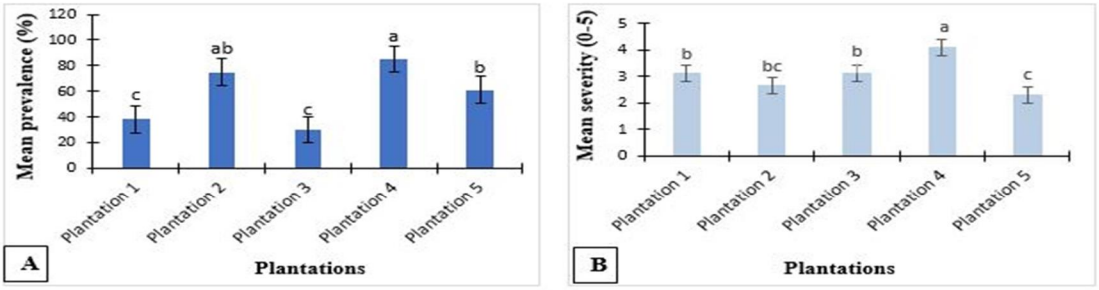
A: Leaf flaccidity; **B**: Chlorosis; **C**: Browning between veins; **D**: Drying of tissues; **E**: Browning of the vascular system

Prevalence of wilt symptoms in tomato plantations:

The prevalence of wilting of tomato plants varied from one plantation to another. The highest mean prevalence (80%) was observed in plantation 4 while the lowest (30%) was recorded in plantation 3 (Figure 3 A). Statistical analysis revealed four different prevalence classes in this area (P ? 0.05).

Severity of wilt symptoms in tomato plantations:

The disease severity varied from 2.3 to plantation 4.63. Statistical analysis revealed four different severity classes in central Côte d'Ivoire (P ? 0.05). The manifestation of the disease was however similar in the plantations 1 and 3 (Figure 3 B).



The bands affected by the same letter are statistically different at 5% threshold.

Figure 3: Mean prevalence and severity of tomato wilt observed in five different plantations in central Côte d'Ivoire A: Prevalence B: Severity

Bacterial strain associated with symptoms:

Three minutes after the introduction of a cut stem of infected tomato into the test tube containing water, whitish threads of bacterial slime streamed from the cortex. The test being positive, it confirms the involvement of *Ralstonia solanacearum* in the induction of bacterial wilt. No other pathogen responsible for vascular plant infection shows this whitish exudate (Figure 4 A).

After seeding the infected tomato explants on SPA medium, whitish to creamy and slightly yellowish colonies were observed (Figure 4 B). This colony coloration on SPA medium is characteristic of *Ralstonia solanacearum.*

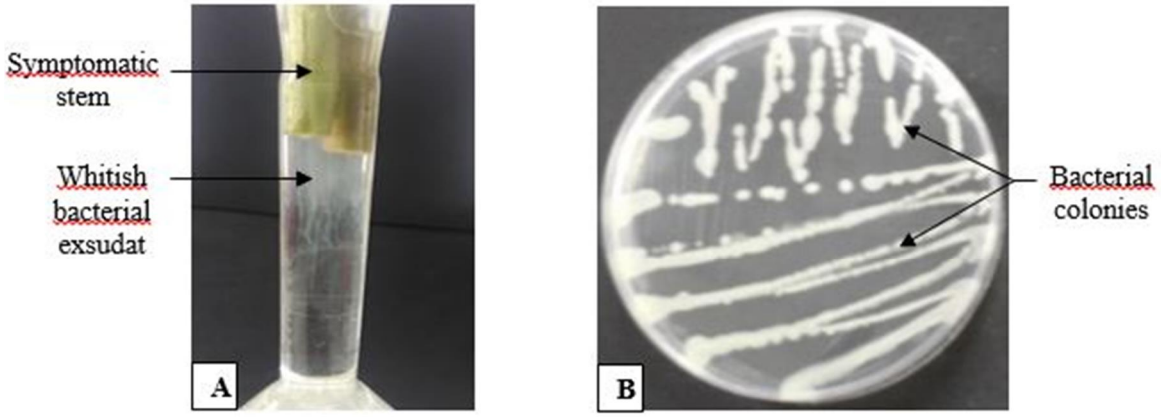


Figure 4: *Ralstonia solanacearum* strain isolated from symptomatic tomato plants
A: Exudation test **B**: 1-day *Ralstonia solanacearum* colony on SPA medium

Pathogenicity of the bacteria *Ralstonia solanacearum*:
Pathogenicity test carried out showed that all tomato plants inoculated with the bacteria *Ralstonia solanacearum* caused symptoms, thus showing the pathogenicity of this bacteria. The symptoms observed were mainly leaf chlorosis, entire plant wilting, drying and death of plants at different times after inoculation (Figure 5).

Figure 5: Tomato plants observed 4 weeks after inoculation with the bacteria *R. solanacearum*.¶
A: Non-inoculated plants B: 3-week inoculated plants C: -week inoculated plants¶
D: 8-weeks inoculated plants.¤

The glass of water or exudation test carried out with the stems of inoculated tomato plants showed the presence of whitish threads in the glass of water, characteristic of the bacteria *Ralstonia solanacearum.* Likewise, this bacteria was isolated from the characteristic symptoms of bacterial wilt. The bacteria *Ralstonia solanacearum* is then the causal agent of the wilt symptoms observed.

Effect of ethanolic extracts of *A. fistulosum* and *H. bonariensis* on *R. solanacearum*:
***In vitro*:**
Ethanolic extracts of *A. fistulosum* and *H. bonariensis* delineated inhibition zones (translucent) at both test concentrations compared to controls (Figure 6).

However, the inhibition diameters of *R. solanacearum* bacteria varied according to the extracts and their concentration. The lowest inhibition diameter (11 mm) was recorded at the concentration of 10 mg / mL of *A. fistulosum* extract while the highest (16.5 mm) was noted at 15 mg / mL of the same extract. Inhibition diameters of the bacterial colony by *H. bonariensis* extract were intermediate (Figure 7). Variance analysis revealed that the antibacterial activity of *A. fistulosum* extract was highest at the 15 mg/mL concentration ($P < 0.05$).

According to Ponce *et al*. (2003) scale, the strain of *R. solanacearum* was highly sensitive to the ethanolic extract of *A. fistulosum* at 15 mg/mL and then sensitive to 10 mg/mL of *H. bonariensis* extract.

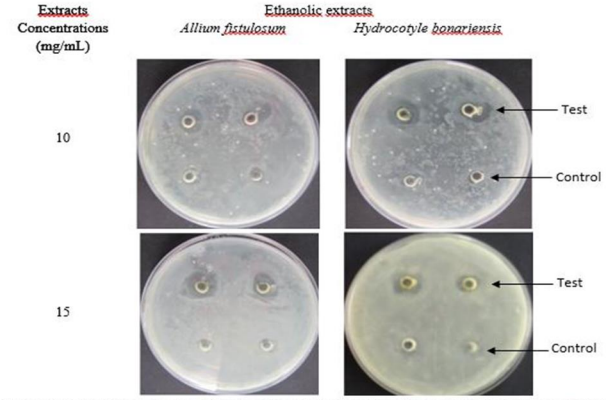
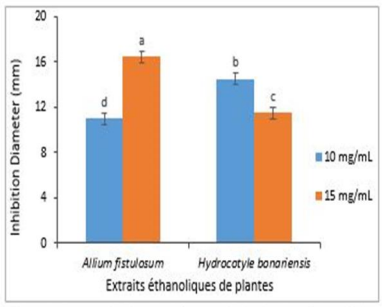


Figure 6: Inhibition zones of *R. solanacearum* colonies by ethanolic extracts of *A. fistulosum* and *H. bonariensis*, 7 days after incubation on SPA medium



Bands with different letters are significantly different at 5% threshold according to Fisher's LSD test

Figure 7: Inhibition diameters of *R. solanacearum* colonies as a function of ethanolic extracts of *A. fistulosum* and *H. bonariensis*, 7 days after incubation

***In vivo*:**

All tomato plants that received the inoculum of *R. solanacearum* bacteria developed symptoms in contrast to healthy control plants. For those inoculated and treated with ethanolic extracts, only low leaves showed symptoms of flaccidity and chlorosis (Figure 8).

Figure 8: Development of tomato plants inoculated with *R. solanacearum* and treated with plant ethanolic extracts, 60 days after inoculation

A: Healthy control plants; B: Inoculated control plants; C: Plants treated with *Allium fistulosum* extract; D: Plants treated with *Hydrocotyle bonariensis* extract

Regarding the prevalence and severity of the symptoms, they varied according to the treatments. Wilting prevalence was higher (58.33%) in inoculated control plants. The symptoms prevalence for inoculated plants treated with extracts of *A. fistulosum* and *H. bonariensis* was low. Thus, wilting prevalence was statistically higher in inoculated control plants than in other treatments ($P < 0.05$), as shown in Figure 9A.

Similarly, wilting was less severe on inoculated plants treated with extracts (average score: 1/5). On the other hand, the disease was severer on inoculated control plants (average score: 2.38 / 5). Wilting severity was significantly higher on the inoculated control plants than on the other plants (Figure 9B).

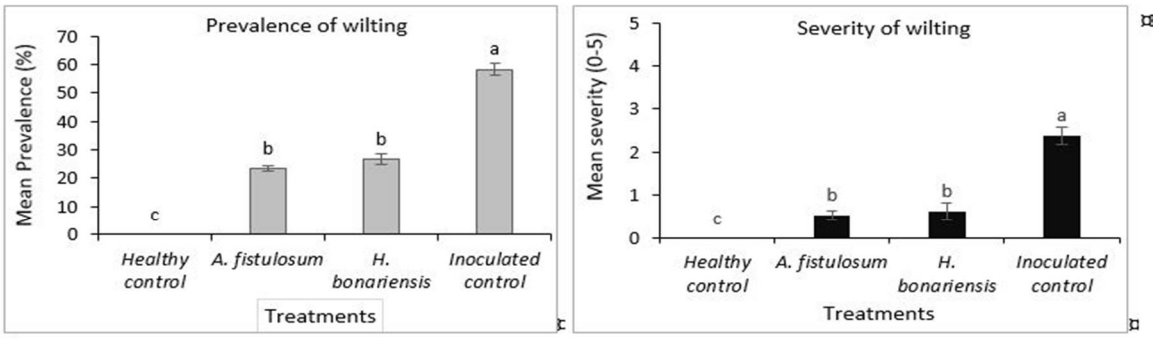


Figure 9: Prevalence and severity of wilting of tomato plants inoculated with *Ralstonia solanacearum* and treated with ethanolic extracts, 60 days after inoculation

A: Prevalence B: Severity

Discussion:

In the course of this study, symptoms of bacterial wilt of tomato were observed in all the plantations visited in the locality of N'gattakro. These include leaf flaccidity, chlorosis, leaf drying and browning of the vascular system. Such symptoms of bacterial wilt of tomato were observed by (Aïssa *et al*., 2014). As a matter of fact, during their work in Niger, these authors also observed browning symptoms of channeling vessel, leaf chlorosis, stunting, leaf flaccidity, and premature leaf loss.

The prevalence and mean severity of bacterial wilt symptoms of tomato at N'gattakro was high. This sensitivity of the tomato to the disease could be due to the variety used. Indeed, Aïssa *et al.* (2014) showed that the Assila tomato variety is susceptible to bacterial wilt disease but resistant to *Fusarium* wilt, *Verticillium* wilt, tomato yellow leaf curl virus (TYLCV) and nematodes. The susceptibility could also be due to climatic conditions. According to Teng *et al*. (2010), the alternating heat of humidity causes the spread of the bacterium *R. solanacearum*, which is responsible for tomato bacterial wilt. Similarly, Cariglia (2007) showed that temperatures above 25 ° C and excess water causes the multiplication and spread of tomato bacterial wilt disease. In addition, Van Elsas *et al*. (2001) and Digat and Caffier (1996) reported that the bacterium can be easily spread by surface water when disease-affected roots develop in stagnant water. According to Messiaen *et al*. (1991) and (Grubben and El Tahir, 2004), bacterial wilt caused by *Ralstonia solanacearum* is a threat to pepper cultivation and other Solanaceae such as tomatoes on contaminated land in tropical countries.

Control of *R. solanacearum* bacteria revealed that ethanolic extracts of *A. fistulosum* and *H. bonariensis* delineated areas by inhibiting colony outbreaks. These extracts further protected tomato plants from wilting by reducing significantly the prevalence and severity of wilting 60 days after inoculation. These antibacterial properties would undoubtedly be due to the phytochemicals in the extracts. Indeed, polyphenols; phytosterols and allicin were reveales in ethanolic extracts of *A. fistulosum* (Vlase *et al*., 2013). Similarly, polyphenols, triterpenes and flavonoids have been identified in *H. bonariensis* extract (Maulidiani *et al*., 2014). Therefore, a synergism between the compounds would confer the antibacterial properties relevant to the extract of each plant. Several studies have shown the antibacterial properties of *Allium* extracts including that of Benmeddour *et al.* (2015). Groshens (2009) also inhibited the growth of R. solanacearum by *A. fistulosum* extract from several tested plant extracts. In the extract of *H. bonariensis*, Eduardo *et al*. (2006) showed the *in vitro* effectiveness of the ethanolic extract of *H. bonariensis* leaves against the bacteria *Bacillus subtilis* "ATCC 6633" and *Pseudomonas aerugino* "ATCC 27853". In the same vein, Abo-Elyousr and Asran (2009) reduced the bacterial wilt index by 50% by *H. bonariensis* extract, when the extract is applied two days before or the same day of inoculation of the bacterium.

Conclusion:

This study shows that wilt symptoms were observed in all tomato plantations visited with prevalences and severities ranging from 30 to 85% and from 2.3 to 4.63 respectively. The symptom-associated bacteria *Raltsonia solanacearum* has reproduced the symptoms of bacterial wilt on the basis of the pathogenicity test. This bacteria was highly sensitive to the ethanolic extract of *A. fistulosum* at 15 mg/mL dose and sensitive to that of *H. bonariensis* at 10 mg/mL dose. At the same doses, the extracts protected the tomato plants against bacterial wilt and caused their seamless development. Field trials could be carried out to integrate the use of extracts from these two plants in the treatment of tomato bacterial wilt.

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