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CENTER FOR RESAERCH AND GRADUATE PROGRAMME IN LIFE SCIENCES-HEALTH AND ENVIRONMENT

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Contribution to the Control of Early Blight, *Fusarium* Wilt, and Damping-off Diseases of Tomato (*Solanum lycopersicum*) Using Plant Extracts

THESIS SUMMARY

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1- BACKGROUND

Phytopathogenic fungi are the causative agents of several diseases of cultivated plants, thus causing enormous crop losses in agriculture. In this regard, the application of synthetic chemical pesticides (or fungicides) is the more widespread pest management strategy to prevent yield and quality losses. The massive and improper use of these fungicides leads to the development of resistant strains among the pathogens. Some of these fungicides may seriously affect human health through the presence of residues frequently detected in fruits and vegetables for human consumption and, additionally, have very often a negative impact on environment. Currently, researches of alternative natural products are intensifying in order to solve these pest issues. Among the non-chemical means of crop disease management, use of phytoextracts is important in view of its eco-friendly nature.

In recent years, Cameroon is emerging as an important producer of vegetables and fruit crops. Among the vegetables, Tomato (*Solanum lycopersicon* L.) is gradually gaining importance as it is grown almost throughout the year for its economic and nutritional value. It is distributed and cultivated in the various bioclimatic zones of the country: its production is over 1.18 million tons harvested on 92626 hectares in 2016 and 1.28 million tons harvested on 105561 hectares in 2017 (Faostat, 2019). Tomato is affected by various phytopathogenic fungi responsible for the most prevalent diseases, namely, early blight, caused by *Alternaria* spp.; damping-off and seedling blights caused by *Pythium* spp., *Rhizoctonia solani* Kuhn and *Verticillium albo-atrum* Reinke & Berthold; *Fusarium* wilt and root rot, caused by *Fusarium oxysporum* f. spp. (Jones e*t al*., 2014). Under favourable climatic conditions, diseases occur in alarming proportions leading to deterioration of yield and quality. Therefore, most strategies for pest management rely almost exclusively on the frequent application of synthetic chemical pesticides during cropping to avoid yield and quality losses. Furthermore, the use of synthetic chemical pesticides to control plant diseases is restricted by their possible carcinogenicity, high and acute toxicity, long degradation periods, and potential environmental pollution. The continuous use of the same available chemicals may have consequences on health, environment, and development of resistant strains among the pathogens (Ishii and Hollomon, 2015; Lucas *et al*., 2015). However, because of consumer concerns about potential health and environmental hazards associated with residues from synthetic chemical pesticides, it is imperative to find new effective strategies that present less risk to human health and the environment. Nowadays, plant-derived products are an interesting and prospective alternative in disease management (Ribera and Zuñiga, 2012; Pusztahelyi *et al*., 2015). Plants (and tropical plants in particular) constitute a rich source of bioactive chemicals for the development of biopesticides as safer disease control agents, as excellently reviewed by Suprapta (2016). For instance, papaya anthracnose (*Colletotrichum gloeosporioides*) can be efficiently managed using tick berry (*Lantana camara*, *Verbenaceae*) extracts (Ademe *et al*., 2013); or grey mold (*Botrytis cinerea*) of blackcurrant was successfully controlled by plant extracts of hyssop (*Hyssopus officinalis*, *Lamiaceae*) and summer savory (*Satureja hortensis*, *Lamiaceae*) (Sesan *et al*., 2015). Therefore, several plants produce an extremely diverse array of low molecular mass compounds, often called ‘natural products’ that play a variety of important roles in the plant. It has been demonstrated that some of these compounds (phenols, flavonoids, quinines, tannins, alkaloids, saponins and sterols) may provide both antimicrobial potential and beneficial

effect on plants, such as early seed germination, plant growth promotion, improved crop yield, and increased resistance to abiotic and biotic stress (Wink, 2010; Almosnid *et al*., 2018; Mekam *et al*., 2019). Additionally, they also enhance postharvest shelf-life of perishable products (Yang *et al*., 2011; Kharchoufi *et al*., 2018; Scavo *et al*., 2019). Phenolic compounds, ubiquitous in plants, are of considerable interest and have received more and more attention in recent years due to their bioactive functions. Polyphenols are amongst the most desirable phytochemicals because of their antioxidant activity. These components are known as secondary plant metabolites and possess also antimicrobial, antiviral and anti-inflammatory properties along with high antioxidant capacity (Ignat *et al.*, 2011).

The search for plant extracts from medicinal plants to control plant pathogens has increased during the last few years. The Cameroonian flora is diversely rich with numerous species of medicinal plants used in traditional medicine since antiquity and commonly present in several Cameroon's regions. Many local plants, such as *E. hirta*, *O. barrelieri*, and *S. cayennensis* have received attention based on their ethnobotanical uses, phytochemical and pharmacological properties, and their capacity to cultivate and harvest easily in tropical areas. *E. hirta* has been chemically studied and found to possess antifungal activity against *Fusarium moniliforme* Sheldon and *Phoma sorghina* Saccardo (Karanga *et al*., 2017). Furthermore, *O. barrelieri* plant extract according to Dakole *et al*. (2016) presents an inhibitory effect on the mycelial growth and conidia germination of *Fusarium oxysporum* f.sp. *lycorpercici* and *Phytophthora infestans* (Mont.) de Bary. Moreover, the plant extract of *S. cayennensis* inhibits the growth of different bacteria (Okoye *et al*., 2010). Nonetheless, tomato fungal pathogens have been mostly neglected so far, and substantial data to control tomato diseases using such plant-based biopesticides are not yet available.

Research hypothesis

The water and hydro-ethanol extracts from selected plant materials, namely, Euphorbia hirta, Oxalis barrelieri, and Stachytarpheta cayennensis are a source of highly effective anti-microbial, antioxidants, plant growth-promoting and protective molecules. They are suitable for use as a promising and environmentally friendly strategy for crop disease control that could contribute to minimizing the risks and hazards posed by conventional synthetic chemical fungicides.

General objective

The general objective of this work was to evaluate the efficacy of water and hydro-ethanolic extracts of E. hirta, O. barrelieri, and S. cayennensis as alternative control measures of damping-off, early blight, and Fusarium wilt diseases of tomato.

Specific objectives

Particularly, our study was:

- To identify, at the species level, two phytopathogenic fungui affecting tomato from Alternaria and Fusarium genus;
- To evaluate the antifungal potential of water and hydro-ethanol extracts of *E. hirta*, *O. barrelieri*, and *S. cayennensis* leaves against mycelial growth of three major

phytopathogenic fungi affecting tomato (*A. solani*, *F. oxysporum*, and *R. solani*) and determine their phytochemical content;
- To identify and quantify the phenolic profile and determine the antioxidant properties of the two different extracts (water and hydro-ethanol) of *E. hirta* leaves;
- To assess the effects of the hydro-ethanol extracts of *E. hirta*, *O. barrelieri*, and *S. cayennensis* leaves on growth-promoting, protection, and fruit production of tomato crop.

2- METHODOLOGY

Among the diseases of tomato, early blight caused by *Alternaria solani*, damping-off cause by *Rhizoctonia solani*, or wilt and rot root cause by *Fusarium oxysporum* are either of the major diseases. Hence, investigation on "Using plant extracts to control *Alternaria*, *Fusarium* and *Rhizoctonia* diseases in Tomato (*Solanum lycopersicum* L.)" was carried out. During present investigation, field surveys were recorded to collect information on the occurrence of disease on tomato in two locations (Zamakoue-barrière and Toutsang) across two regions in Cameroon. Laboratory studies on Morphological and molecular variability among the *Alternaria* sp and *Fusarium* sp isolates; efficacy of plant extracts against *Alternaria solani*, *Rhizoctonia solani*, and *Fusarium oxysporum* f. sp. *vasinfectum*; phytochemical content and polyphenol profiles of the plant extracts; and plant defense enzyme activities analyses were undertaken. Further, the potential of ethanol extracts to improve growth promotion, disease reduction, defense system stimulation, and fruit yields of tomato were assessed under greenhouse and field conditions.

2-1- Morphocultural and molecular identification of *Fusarium oxysporum* sp. and *Alternaria* sp. isolated from infected tomato plant organs

In present study, identification of *Fusarium oxysporum* sp. and *Alternaria* sp isolated from tomato fields in two crop production areas in Cameroon was done based on (1) morphological characters like the colony aspect, colour, growth rate, size and shape of macro- and micro-conidia according to the criteria described by Leslie and Smmerell (2006) and Van der Waals *et al*. (2004), respectively; and (2) genetic diversity of Internal Transcribed Spacer (ITS) sequences of rRNA and partial Translation Elongation Factor 1α (TEF-1α) gene by using PCR techniques.

2-2-Evaluation of the plant extracts antifungal activity

To investigate the direct antifungal effects of plant extracts, the efficacy of water and ethanol extracts of tested tropical plants against *F. oxysporum*, *A. solani* and *R. solani* was assessed *in vitro* using the supplemented agar plate method described by Rios *et al*. (1988).

2-3- Analysis of phytochemical content of plant extracts

Preliminary analyses of water and ethanol extracts of *Oxalis barrelieri*, *Stachytarpheta cayennensis* and *Euphorbia hirta* were performed to assess their phytochemical coontent. Each extract was analyzed for: i) total phenolic content using the method of Singleton *et al*. (1999), ii) total flavonoid content using the method of Zhishen *et al*. (1999), iii) total tannin content using method of Verzelloni *et al*. (2010), iv) total alkaloid content with the method of Tabasum and Khare (2016), v) total polysaccharide content using the method of Dubois *et al*. (1956), and vi) total protein content using the method of Bradford (Bradford, 1976).

2-4- Analysis of phenolic compounds profile of water and ethanol extracts of *Euphorbia hirta* leaves

The phenolic compounds profile of extracts was analyzed using an un-targeted method through LC-ESI-IT-MS/MS experiments as reported in Mena *et al*. (2016).

2-5- Analysis of antioxidant activity of water and ethanol extracts of *Euphorbia hirta* leaves

The antioxidant properties of water and ethanol fractions obtained from *E. hirta* leaves were analyzed by using four different assays. The radical scavenging ability was analyzed using the ABTS assay (Re *et al*. (1999). The reducing ability was tested by using the protocol based on the ferric reducing/antioxidant power (FRAP) assay (Benzie and Strain, 1999). The capacity to scavenge superoxide anion radicals was evaluated using the method of Martini *et al*. (2017). The chelation of ferrous ions or Fe^{2+}-chelation ability was instead evaluated by the ferrozine assay, using the method of Le *et al*. (2007) (Karama and Pegg, 2009).

2-6- Evaluation of growth promotion and protective effects of the *Euphorbia hirta* ethanol extract leaves under greenhouse conditions

Ethanol extract of *Euphorbia hirta* leaves (EE*eu*) was chosen to perform Greenhouse *in planta* experiments, for assessment of plant growth effect and protective effect against *R. solani*. An aqueous solution of EE*eu* was sprayed onto the shoots canopy of tomato plants at a concentration of 2.50 $mg.mL^{-1}$. Inoculation was carried out 48 hours after by transferring the tomato plants into new pots containing infested soil-perlite (3:1, v:v). The soil was infested by setting 2.50 g of *R. solani* inoculum paste at the bottom of a hole made at mid-depth in each pot (Logemann et al., 1992). For each treatment studied, the effects of EE*eu* on the tomato plant growth was evaluated by measuring the height of plants at 7, 14, and 21 days after extract application. Then, on inoculated tomato plants, *Rhizoctonia* disease severity was scored base on a 0-5 disease scale according to Weitang *et al*. (2004) and used to calculate disease severity and disease reduction percentages at 5, 10, 15, and 20 days after inoculation.

2-7- Evaluation of defense-related enzyme activities in tomato leaves

The experiment consisted to measure in tomato treated with ethanol extract of *Euphorbia hirta* some specific defense-related enzymes, known as antioxidant enzymes and considered as makers correlated to the development of induced disease resistance, over a time course by using spectrophotometric dosage technics. Catalase activity was measured using the method of Havir and Mc Hale (1987). Superoxide dismutase was determined using the indirect method SOD determination Kit-WST (Sigma). Chitinase activity was assayed as described by Boller and Mauch (1988). Phenylalanine ammonia-lyase (PAL, E.C.4.3.1.2.4) activity was assayed as described by Ross and Sederoff (1992).

2-8- Evaluation of fruit yield on tomato treated with ethanol extracts under field conditions

Field *in planta* experiment was conducted for assessment fruit yield of tomato plants treated with ethanol extracts. Fruit yield assessments were carried out after the end of the

treatments by picking the fruits at the time of ripening (end of July and mid-August). The fruits from each plant across all of the treatments were separately harvested at 90 and 121 days after transplantation, respectively, for the (i) healthy (commercial), (ii) fungal and (iii) physiological symptomatic tomatoes. Both of these sets of data were calculated as the means of 20 plants per treatment, then brought back to a plant and reported per hectare after Vanounou (1997) to estimate the potential fruit yield.

2-9- Statistical analyses

Mass spectrometry, antioxidant activity, and enzyme activty data are displayed as means ± standard divisions for three replicates for each prepared sample. Antifungal activity data are reported as means ± standard division for five replicates. Univariate analysis of variance (ANOVA) with Tukey's post-hoc test was applied using GraphPad 9 prism 6.0 (GraphPad Software, San Diego, CA, U.S.A.) when multiple comparisons were performed or to evaluate whether extracts and concentrations had effect on the fungal growth. The differences were considered significant with $P < 0.05$.

3- RESULTS

3-1- Fungus identification

In general, there were some variations between cultures grown on PDA media and V8 media. Among the isolates, isolate 2 expressed the best myceliar growth on V8 medium (8.89 $mm.day^{-1}$) compared to PDA mediun (8.33 $mm.day^{-1}$). The isolate 1 shown colony with white and brown fluffy aerial mycelium and brown on the reverse. Conidia were grown from monophialid. Macroconida were brown, ovoid and straight, apical basal cell, with 3-5 longitudinal septates and 2-3 transversal septates. Microconidia were abundant, unicellular, and round. The morphology description of this fungus agreed with the characteristics of *Alternaria* genus which cause tomato early blight. The isolate 2 shown colony with white cottony aerial mycelium and purple on the reverse. Conidia were grown from short phialid with a false head. Macroconida were straight fusiform or slightly curved, pedicellate basal cell, with 3-5 septates. Microconidia were abundant, ellipsoid or fusiform without or with 1-2 septates. Chlamydospores were formed terminally or intercalary, single or in pairs. The morphology description of this fungus agreed with the characteristics of *Fusarium* genus which cause tomato wilt and root rot.

Molecular identification using the data base of the NCBI GenBank (http://ncbi.nlm.nih.gov/BLAST/) revealed that the two isolates, previously identified as belong *Alternaria* spp. and *Fusarium* spp., were closely related (99 % homology) to *Alternaria solani* and *Fusarium oxysporum* f.sp. *vasinfectum*, respectively.

3-2- Antifungal activity of plant extracts

Growth of the three phytopathogenic fungi on PDA medium, without addition of plant extracts, reached the following mean colony diameters after one week: 8.50 cm for R. solani, 7.50 cm *for F. oxysporum* f. sp. *vasinfectum* and 7.38 cm for *A. solani*.

In general, extracts from *E. hirta* gave greater inhibition than those from *S. cayennensis*, which were more active than the extracts from *O. barrelieri*. The ethanol extracts gave greater antifungal activity than the water extracts. As little as 1.25 $mg.mL^{-1}$ of ethanol extracts from the all three plants were sufficient to inhibit fungus growth by 10 to 28% (depending on fungus). Between 10 to 20 $mg.mL^{-1}$ of ethanol extract, growth inhibition

was 90-100%. At low doses, water extracts from all three plants also inhibited fungus growth by 2 to 28% (depending on fungus) but, differently from the ethanol extracts. Growth inhibition from ethanol extracts were two- to three-fold greater than for water extracts at equivalent concentrations.

3-3- Phytochemistry of plant extracts

Yield and colour characteristics of plant extracts varied from solvent used during the extraction procedure and plants species. WE*ox* and EE*ox* from *O. barrelieri*, with an average yield of 6.22 and 5.14 % of initial leaf biomass; WE*st* and EE*st* from *S. cayennensis*, with an average yield of 7.35 and 5.25 %; and WE*eu* and EE*eu* from *E. hirta*, with an average yield of 8.20 and 5.60 %, respectively. Water was able to extract more substance from leaves than hydro-ethanol solution in the three plant species.

Except for the alkaloids, whose contents in plant extracts were almost the same, the quantification of chemical components gave different values in all tested plant extracts. EE*st* displayed the major content for: phenolics (101.70 ± 11.80 mg gallic acid. g^{-1}), flavonoids (33.50 ± 0.70 mg catechin. g^{-1}), and tannins (7 ± 1.90 mg catechin. g^{-1}). WE*ox* had the major content of proteins (8.10 ± 0.80 mg BSA.g^{-1}). The polysaccharide components were higher in water extracts compared to the ethanol extracts, except in EE*st* (179 ± 27.30 mg glucose. g^{-1}) and WE*st* (94.80 ± 18.90 mg glucose. g^{-1}).

3-4- Phenolic compounds profile and antioxidant property of water and ethanol extracts of *Euphorbia hirta* L. leaves

LC-ESI-IT-MS/MS allowed the tentative identification of 123 phenolic compounds, 7 organic acids, 4 terpenes, 3 amino acids, 1 dipeptide; 1 alkaloid; 1 anthraquinone and 1 norisoprenoid. Water extract of *E. hirta* leaves contained more phenolic compounds than the ethanol extract 163.62 ± 0.61 mg.g^{-1} of dry extract versius 49.61 ± 0.39 mg.g^{-1} of dry extract ($P< 0.05$), respectively. Water extract was particularly rich in gallotannins and hydroxybenzoic acids (representing the 31.4 % and 26.5 % of total phenolic compounds, respectively) whereas the ethanol extract was enriched in hydroxycinnamic acids and isocoumarins (representing the 45% and 16.7% of total phenolic compounds, respectively). The ethanol extract of *E. hirta* leaves was more effective, with respect to the corresponding water extract; in scavenging ABTS ($P< 0.05$) and superoxide anion radicals ($P< 0.05$). Furthermore, the ethanol extract also showed higher reducing power with respect to the water extract ($P< 0.05$). On the other hand, the water extract exhibited better chelating ability towards Fe^{2+} than the ethanol extract ($P< 0.05$).

3-5- Tomato plant height, disease reduction, and inducing plant defense-related enzymes with *Euphorbia hirta* ethanol extract treatment

In greenhouse experiments, spraying tomato plants with hydro-ethanol extract of *E. hirta* at 2.50 mg.mL^{-1} increased plant size when compared to untreated plants. Spraying tomato plants with hydro-ethanol extract of *E. hirta* and infected by *R. solani* laid to increase of catalase, superoxide dismutase, chitinase, and phenylalanie amino-lyase activities and reduced disease severity up to 80% when compared to non-sprayed plants.

3-6- Tomato fruits production with hydro-ethanol extracts of *Euphorbia hirta, Oxalis barrelieri,* and *Stachytapherta cayennensis*

In field conditions, spraying tomato plants with hydro-ethanol extracts of *E. hirta, O. barrelieri,* and *S. cayennensis* increased fruit production compared to the plants sprayed with water. Highest production of total marketable fruits (33 ± 6 number.plant^{-1} ; 60 ± 9 kg.20 plants^{-1}; 45.07 ± 9.75 T.ha^{-1}),.and Smallest mass lossed of fungal symptomatic fruits (7.72 ± 0.87% of spoiled fruit rate) and physiological symptomatic fruits (5.87 ± 1.11% of spoiled fruit rate) were recorded on the tomato sprayed with *E. hirta* ethanol extract.

CONCLUSION

In the light of present investigation, which focused on contribution to the control of early blight, Fusarium wilt, and damping-off of tomato by using plant extracts, it follows that:

On the basis of morphocultural and molecular variabilities observed among the isolated tomato fungal phytopathogenics, Alternaria solani and Fusarium oxysporum f. sp. vasinfectum were identified and appear as the major pathogens of tomato from Toutsang and Zamakoué-barrière localities.

Water and hydro-ethanol extracts obtained from *Euphorbia hirta*, Oxalis barrelieri, and Stachytapherta cayennensis leaves demonstrated a potent inhibitory activity on some tomato pathogenic fungi. Among the six plant extracts, the most inhibitory were hydro-ethanol extracts of *E. hirta, O. barrelieri,* and *S. cayennensis*. Therefore, they can be used to develop natural products for the biocontrol of *Rhizoctonia solani* damping-off disease, *A. solani* early blight disease, and *F. oxysporum* f. sp. *vasinfectum* wilt disease.

Water and hydro-ethanol extracts obtained from *E. hirta, O. barrelieri,* and *S. cayennensis* leaves are sources of phytochemicals that were rich in polyphenolic constituents and demonstrated the strong antioxidant abilities. The phenolic profile of water and hydro-ethanol extracts of *E. hirta* leaves identified and quantified were rich in phenolic classes as hydroxybenzoic acids, hydroxycinnamic acids or isocoumarins, and in bioactive components specially tri-O-galloyl-glucose isomers and feruloyl-coniferin.

Foliar application of *E. hirta*, *O. barrelieri*, and *S. cayennensis* hydro-ethanol extracts had promoted the plant growth and reduced symptoms cause by *Rhizoctonia solani* on tomato, and increased the tomato production under greenhouse and open field conditions. Thus, the use of these plant extracts can constitute and alternative method to conventional fungicides since they are ecofriendly agrochemicals for crop disease control that could contribute in minimizing the risks and hazards of toxic fungicides.

PERSPECTIVES

Based on the results and conclusions of this study, the following perspectives are suggested for further reseach devoted to:

- The study of nutritional composition and biological properties of treated tomato fruits.
- The study of gene expression for a better understanding of the mechanisms by which E. hirta extract affects tomato by reducing early blight, Fusarium wilt, and damping-off diseases. Since the tomato genome is fully sequenced and putative defense genes are known, there are the best prerequisites for these investigations. As discussed before, this would ideally complement phenotypical and biochemical orientated experiments as conducted in this thesis.

- The evaluation of economic advantages that may motivate to growers applying these plant extracts as botanical biopesticides and stimulants. Economic advantages from the use of plant extracts could be particularly relevant in African rural areas, where these pan-tropical plants are common and adapted. Biomolecules may be developed into commercial products by local companies, thus contributing to rural and agro-industrial development, together with increased sustainability for local cropping systems.

PUBLICATIONS

Mekam P.N., Martini S., Nguefack J., Tagliazucchi D., Mangou G. N., E. Stefani, (2019). Activity of extracts from three tropical plants towards fungi pathogenic to tomato (*Solanum lycopersicum*). *Phytopathlogia Mediterranea*. 58 (3), 573-586. Doi:10.13128/Phyto-10891.

Mekam P.N., Martini S., Nguefack J., Tagliazucchi D., E. Stefani, (2019). Phenolic compounds profile of water and ethanol extracts of *Euphorbia hirta* L. leaves, showing antioxidant and antifungal properties. *South African Journal of Botany*. 127, 319-332. https://doi.org/10.1016/j.sajb.2019.11.001.