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In Vitro Activities of Essential Oils and fractions of Two Cameroonian Plants against Tomato Wilt Fusarium oxysporium lycopersici.

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Abstract

In this study we surveyed the effectiveness, Minimum inhibitory concentration (MIC) and Minimum fungicide concentration (MFC) of essential oils from two medicinal plant *Callistemon citrinus* and *Tephrosia*. *vogelli* on mycelial growth of tomato pathogenic fungi *Fusarium oxysporium lycopersici*. Oils were extracted using a Clevenger apparatus and fractions with column chromatography combine to thin layer chromatography. The rates of inhibition growth were measured after placing active mycelial plugs of the fungi on Petri dishes containing PDA amended with specific concentrations of crude essential oils or fractions from each plant at 37ºC for 7 days. Data were analyzed using MSTATC and SAS (version 9.1.3) software. Essential oils extractions were effective with yield rate of 0.4 and 0.7 for *Tephrosia*. *Vogelli* and *Callistemon citrinus* respectively. These essential oils inhibited our fungi with growth inhibition average of 100% at 1µl/ml and 6µl/ml concentration for *Tephrosia*. *Vogelli* and *Callistemon citrinus* respectively. Fractionation of these oils gives 10 and 8 fractions for *Tephrosia*. *Vogelli* and *Callistemon citrinus* respectively. From the 8 fractions obtain with *Callistemon citrinus* oil, 3 totally inhibit fungi growth at 4 and 5 µl/ml concentration respectively, while from the 10 obtain with *Tephrosia*. *Vogelli* oils, 3 also shown total inhibition of the fungi growth at 0.5 and 0.8 µl/ml concentration respectively. At 2µl/ml, crude oils and fractions 2, 3 and 4 of *Callistemon citrinus*, were more active than the synthetic antifungi Callomil and less than Manebra, however fraction 4 and 5 of *Tephrosia Vogelli* were as active as Manebra and less active than callomil. Subculture analysis shown that fraction 4 of *Callistemon citrinus,* fraction 4, 5 and crude oil extract of *Tephrosia Vogelli* plant exhibit fungicidal properties while the rest exhibit fungi static properties. Since growth inhibition of studied essential oils and fractions was evident in this study, they have potential to control plant disease due to *Fusarium oxysporum lycopersici* and could be considered for developing new fungicides.

Key words: essential oils; Tephrosia Vogelli; Callistemon citrinus; Fusarium oxysporium lycopersici; tomato; column chromatography.

Introduction

Incomes from agriculture are in great importance for the economics of many countries across the world. Tomato (Lycopersicon esculentum) production was estimated at 113,308 billion (FAO, 2004). This plant is of great importance for Small scale producer economy in many tropical countries across the world. Tomato producers suffered immense looses due to the diseases in many countries of the world (Walker, 1971; Volin and Jones, 1982). Fusarium oxysporum is a cosmopolitan fungus that can be found in soils in all part of the world. The fungus is known to produce sparse to abundant aerial mycelium, and white, pink, salmon and purple pigmentation on the reverse side of the colony in culture (Nelson et al. 1983; Gerlach and Nirenberg, 1982). It can be divided into many formae speciales most of which attack a single crop system (Kistler, 1997). Fusarium wilt is a warm-weather disease, most prevalent on acid soils, which causes highly destructive diseases in many economically important agricultural crops like tomato. It causes a vascular disease that is commonly known as wilt, root rot, or yellows (Talboys 1972; Beckman and Roberts 1995). Dissemination of the pathogen is via seed, tomato stakes, soil, and infected transplants or infested soil adhering to transplants. Although resistant cultivars are known to exist, the occurrence and development of new races is a continuous problem (Borrero et al. 2006). Pasteurization of infested soil with steam or fumigants, raise the soil pH to 6.5-7.0, and usage of nitrate nitrogen rather than ammonium nitrogen help to reduce the incidence of wilted plants and greatly increases marketable and total yields (Borrero et al. 2006). Another way to overcome this problem is the development of fungicides with natural plant metabolite origins. There is no doubt that plants are a reservoir of potentially useful chemical compounds which serve as drugs, provided newer leads and clues for modern design by synthesis (Evans, 2002; Varier, 1995). In fact, many studies shown that herbal and tree extracts have potential antimicrobial actions (Rahma et al., 2013; Manaheji et al. 2011; Atawodi et al., 2010; Singh et al., 2010; Hossain et al., 2006; Jazet et al., 2002).

Tephrosia vogelii is a perennial small leguminous shrub with mauve flowers, many pods and of about three to four meters long. Its well known properties are ichthyotoxic, insecticidal, food parasiticidal, antifeedant, antibacterial, antifungal and pest crops control (Teugwa et al. 2013; Ibrahim et al., 2000; Wanga et al., 2000). Several isoflavonoids have been isolated from Tephrosia species with different effects ranging from anti-feedant, antibacterial, insect-repellant and insecticidal (Machocho et al., 1995).

Callistemon citrinus known as bottlebrush perfectly describes this evergreen plant's bright red flower spikes. The flowers are followed by small woody capsules that look like bead bracelets on the bark and which last for years. Many studies have been done on medical properties of different species of Callistemon citrinus. Aqueous extracts of the leaves and flowers have antifungal, antibacterial and cholinesterase activity. The essential oils from leaves possess antimicrobial, fungitoxic, antinociceptive and anti-inflammatory activities (Kumar et al., 2011, Jazet et al., 2009, Anudwipa et al. 2008). Until known, those important compounds are well study on human pathogen however information on their activity on plant pathogens is scarce. This work aims at study the antifungal properties of essential oils from two medicinal plant Callistemon citrinus and Tephrosia. vogelli on a tomato pathogenic fungi Fusarium oxysporium lycopersici. Specifically we surveyed the effectiveness, Minimum inhibitory concentration (MIC) and Minimum fungicide concentration (MFC) of essential oils and fractions from the two plants on the mycelial growth of a tomato pathogenic fungi Fusarium oxysporium lycopersici afterward we determine the fungi-static and fungi-toxicity properties of those extracts.

Materials and methods

Plant samples

Leafs of Callistemon citrinus collected in the campus of Yaoundé I University in March, 2005 and Tephrosia. vogelli collected in the same periods at Mbouda sub-division in the west region of Cameroon were used in this study. All samples were authenticated at the National Herbarium of Cameroon (Yaoundé) where specimens were deposited under the reference numbers: N°10356/SRF/CAM for Callistemon citrinus and N°24450/SRF/CAM for Tephrosia. vogelli.

Fungi isolate

Fusarium oxysporum lycopersici, obtained from the Laboratory of Plant Pathology of the National Institute of Agricultural Research for Development in Cameroon was used to study the antifungal activities of oil extracts and fractions. The so obtained fungi was successively inoculated twice in PDA medium and allow for growth at 25°C for a week for activation before used for the experiment. Within the growth, colonies shown structure characteristics of Fusarium oxysporum fungi 6 days after inoculations as describe in the previous work of Mui Yung Wong, 2003.

Plant extraction and fractionation

Plant materials were previously dry at room temperature (30°C). 300 g of the air-dried aerial parts of the plants were hydro-distilled for 6 consecutive hours using a Clevenger-type apparatus (Britania pharma-copeia model). The obtained essential oils were dehydrated over anhydrous sodium sulphate, filtrated and stored at 4°C until tested (Sahin et al., 2004). Extraction yield were evaluated using the formula: % = essential oils weight x 100/plant material weight. The obtained extracts were further used for fractionation and antifungal test as follows.

Part of essential oils solution was mixed with silica gel and chromatographed on a silica gel column by elution with gradients of Hexane, EtOAc and MeOH range as follows: Hexane 100%; Hex- EtOAc, (95-5, 90-10, 75-25, 50-50, 25-75); EtOAc 100%; EtOAc-MeOH, (55-45) and MeOH 100%. An aliquot of 100 ml of eluate was collected for each fraction. TLC analyses were used to monitor the chemical composition of each fraction. Fractions with similar compositions were pooled to 10 fractions for Tephrosia. vogelli and 08 fractions for Callistemon citrinus (EA-1 to EA-45).

Antifungal activity assay

Agar dilution method was used for minimum inhibitory concentration (MIC) and minimum fungicidal concentrations (MFC) determination (Berghe and Vlietnick, 1991). Plant extracts and fractions were first diluted With dimethylsulfoxyde(DMSO) in a proportion of 1/9 and then to drug-free agar medium (SDA), resulting in final concentrations ranging from 0,5 to 6 µl/ml. Explants of 7 mm diameter were seeded in triplicate at the centre of the Petri dish that was sealed and incubated at 37°C for seven days. Drug-free SDA was prepared, inoculated and incubated in the same condition as negative control. Callomil and manebra at 0.4mg/ml and 0,33mg/ml was used as positive control. The growth diameters were subsequently measured and the inhibition rate (I) calculated as follows:

%I = (Dc-DtX100)/Dc.

(Dc = growth diameter records for control, Dt = growth diameter records for test).

The lowest concentration at which no growth was observed was considered as the MIC. Petri dish in which no fungal growth was observed was sub-cultured on drug-free agar medium (SDA). The dishes were incubated at 37°C for 7 days. The lowest concentration exerting complete visible growth inhibition was considered as the MFC while other was fungi statics.

Statistical analyses

The results are presented as means ± standard deviation (SD). Data were analyzed using the statistical package for social sciences (SPSS) 10.1 software for Windows. The mean values were compared using Student-Newman-Keuls test with $p < 0.05$.

RESULTS

Essential oils extraction from Tephrosia vogelii and Callistemon citrinus plants exhibit high yield value for callistemon citratus.

Table: 1 Yield of essential oils extractions of two Cameroonian plants Tephrosia vogelii and Callistemon citrinus.

Samples	Yields of extraction	Colours
Tephrosia vogelii	0,4	Yellow
Callistemon citrinus	0,7	Yellow

The antimicrobial effect of Callistemon citrinus and Tephrosia vogelii essential oils at various concentrations ranging from 0,5 to 6 µl/ml was evaluated after 7 days of growth of the fungi Fusarium oxysporum lycopersici (table 2). The effects of five concentrations tested showed significant difference at $p \leq 0.01$ for the two oils. Tephrosia vogelii essential oil was the most active because it completely inhibits growth of the microorganism up to 7 days at 1µl/ml concentration only and more, while Callistemon citrinus was a weak inhibitor as it completely inhibits microorganism growth up to 7 days at 6µl/ml concentration. More than 50% of growth of the fungi was observed at 2µl/ml concentration for Callistemon citrinus essential oils.

Table: 2 Inhibition percentage of Fusarium oxysporum lycopersici gowth by essential oils from Callistemon citrinus and Tephrosia vogelii 7 days after incubation.

Essential oils (µl/ml)	Inhibition rate (%)	
	Callistemon citrinus	Tephrosia vogelii
0,5	15,7e	79,64b
1	42,5d	100a
2	60,3c	100a
4	80b	100a
6	100a	100a

Data in the same column follow by the same letters are not significantly different at 5%

Further fractionation with analytical Tin Layer Chromatography guide of our essential oils yield 8 and 10 different fractions for Callistemon citrinus and Tephrosia vogelii respectively. Fractions were codified as CcF1…8 for Callistemon citrinus and TvF1…10 for Tephrosia vogelii.

Antifungal activity test of the 8 Callistemon citrinus essential oil fractions were now tested against the fungi Fusarium oxysporum lycopersici at 4 different concentrations ranging from 2 to 6µl/ml (table 3). Significant differences were observed between the five concentration tested for the various fractions at p ≤ 0.01. Amongst the 8 fractions, CcF2, CcF3and CcF4 was the most active because they totally exhibit total inhibition of the fungi at 5 and 4µl/ml concentrations respectively for fraction 2 and fraction 3-4. CcF1, 5, 6, 7, and 8 were weak inhibitors because they exhibit inhibition of microorganism growth for 7 days at 6µl/ml only.

Table: 3 Inhibition percentage of Fusarium oxysporum lycopersici gowth by essential oils fractions from Callistemon citrinus 7 days after incubation.

Fractions	Essential oils (µl/ml)			
	2	4	5	6
CcF1	Nt	Nt	Nt	50,7e
CcF2	70,74d	85,60b	100a	100a
CcF3	80,12c	100a	100a	100a
CcF4	85,7b	100a	100a	100a
CcF5	Nt	Nt	Nt	70,83d
CcF6	Nt	Nt	Nt	82,63bc
CcF7	Nt	Nt	Nt	17,36f
CcF8	Nt	Nt	Nt	2,08g

Data in the same line follow by the same letters are not significantly different at 5%

Nt mean that we did not reach total inhibition of our fungi growth within the tested concentrations.

Antifungal activity test of the 10 Tephrosia vogelii essential oil fractions against the fungi Fusarium oxysporum lycopersici at 4 different concentrations ranging from 0,4 to 1µl/ml (table 4). Results reviled that significant differences exist between the five concentrations tested for the various fractions at p ≤ 0.01. Amongst the 10 fractions, TvF3, TvF4 and TvF5 was the most active because they exhibit total inhibition of the fungi at 0,8 and 0,5µl/ml concentrations respectively for fraction 3 and fraction 4-5. TvF1, 2, 6 - 10 were weak inhibitors because they exhibit inhibition of microorganism growth for 7 days at 6µl/ml only. Except TvF9, all the weak inhibitor fractions exhibit more than 50% of action on fungi growth for 7 days at 1µl/ml concentrations.

Table: 4 Inhibition percentage of Fusarium oxysporum lycopersici gowth by essential oils fractions from Tephrosia vogelii 7 days after incubation.

Fractions	Essential oils (µl/ml)			
	0,4	0,5	0,8	1
TvF1	Nt	Nt	Nt	55,55f
TvF2	Nt	Nt	Nt	82,03c
TvF3	82,37c	87,78b	100a	100a
TvF4	89,97b	100a	100a	100a
TvF5	98,92a	100a	100a	100a
TvF6	Nt	Nt	Nt	70,83e
TvF7	Nt	Nt	Nt	77,77d
TvF8	Nt	Nt	Nt	46,18g
TvF9	Nt	Nt	Nt	20,48h
TvF10	Nt	Nt	Nt	68,40e

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Data in the same line follow by the same letters are not significantly different at 5%

Nt mean that we did not reach total inhibition of our fungi growth for the tested concentrations.

Crude extract and fractions of essential oils with more than 50% activity were now tested in comparison with standard (Manebra and Callom II) for their Antifungal activity. The working concentration was 2µl/ml for Callistemon citrinus and 0,5µl/ml for Tephrosia vogelii essential oil (table 5). None of the crude essential oils of the two plant exhibit total inhibition of the fungi as did Manebra however all the two extracts were more active than Callomil Plus at the tested concentrations for 7 days. In revenge, TvF4 and 5 totally inhibit fungi growth for 7 days as did Manebra while TvF3 inhibit at 85, 34 % in the same condition. None of the 3 Callistemon citrinus essential oils fractions exhibit total inhibition action on the fungi like Manebra but all the fractions were more active than Callomil Plus.

Table: 5 Inhibition percentage of Fusarium oxysporum lycopersici gowth by standard, essential oils and fractions from Callistemon citrinus and Tephrosia vogelii 7 days after incubation.

Callistemon citrinus		Tephrosia vogelii	
Essential oils (2µl/ml)	Inhibition rate (%)	Essential oils (0,5µl/ml)	Inhibition rate (%)
Crude oil	60e	Crude oil	81,30c
CcF2	70d	TvF3	85,34b
CcF3	76,30c	TvF4	100a
CcF4	83,10b	TvF5	100a
Manebra	100a	Manebra	100a
Callomil	55f	Callomil	42,12d

Data in the same column follow by the same letters are not significantly different at 5%

Subculture analysis from dishes used for activity test of crude oils and fractions were made and results presented in table 6. Crude extract and fractions 2 and 3 of Callistemon citrinus essential oils exhibit fungi static activity at concentration of 6, 5 and 4 µl/ml activity respectively while fraction 4 exhibit fungicidal activities at 4 µl/ml concentration. Elsewhere, crude extract and fractions 4 and 5 of Tephrosia vogelii essential oils exhibit fungicidal activities at 1, 0.5 and 0.5 µl/ml concentrations respectively while fraction 3 exhibit fungi static activities at 0.8µl/ml concentration.

Table 6. sub culture analysis of the activity of the tested oils and fractions on the pathogen Fusarium oxysporum lycopersici after 7 days of incubations.

Callistemon citrinus			Tephrosia vogelii		
	CMI/CMF	Qualifications		CMI/CMF	Qualifications
Crude oil	6	Fungi static	Crude oil	1	Fungicidal
CcF2	5	Fungi static	TvF3	0.8	Fungi static
CcF3	4	Fungi static	TvF4	0.5	Fungicidal
CcF4	4	Fungicidal	TvF5	0.5	Fungicidal

Discussion.

The antimicrobial activities of various plants have been reported by numerous studies since a long time. Plants produce secondary metabolites in order to protect themselves from microorganism, herbivores and insects, thus antimicrobial effect is somehow expected from them. Those metabolites are obtained through organic solvent extractions or hydro distillation leading to essential oils. Both extracts rich in secondary metabolites like flavonoids, alkaloids, triterpenoid are producing a better opportunity for testing wide range of microorganism. In the present study a variety of plant pathogenic fungi Fusarium oxysporum strains was used to screening antifungal effects of essential oils of Callistemon citrinus and Tephrosia vogelii. Results showed that Callistemon citrinus yielding more oil than Tephrosia vogelii. This difference can be attributed to genetic factors governing plant distribution, growth and metabolism. In contrast, Tephrosia vogelii essential oils exhibit better inhibition rate against our test fungi than Callistemon citrinus as growth was totally inhibited at 1µl/ml with Tephrosia vogelii oil and at 6µl/ml with Callistemon citrinus oil (table 2). Many study point out the action of plant extracts including essential oils on growth inhibition of a variety of microorganisms with various level of activities related to plant species (Talarat Sookto, 2013; Teugwa et al. 2013; Nafiseh et al., 2011; Ibrahim et al., 2000; Wanga et al., 2000) Gaz chromatography analysis showed more diverse and complex composition of Tephrosia vogelii compare to Callistemon citrinus. Such differences could partly explain the observed activity. In other to better understand the previous observation, we initiated a bio-oriented fractionation of those oils and results showed that Callistemon citrinus allow us to 8 fractions, with varied range of antifungal activity against the tested microorganism. Three of the obtained fractions totally inhibit the mycelia growth of our fungi 7 days after incubation at the concentration of 4µl/ml for fraction 3 and 4, and 5 µl/ml for fraction 2 (table 3). These activities could be attributed to chemicals present in oils from this plant. Previous study on Callistemon citrinus essential oils showed inhibition properties against a fungi Phaeoramularia angolensis and this activities was closely related to its chemical compositions, precisely the presence of 1,8-cineole, α -terpineol and terpinen-4-ol (Jazet et al., 2009). In the other hand, Tephrosia vogelii, give right to 10 fractions, with varied range of antifungal activity against the tested fungi. Three of those fractions also exhibit total inhibition rates on mycelia growth of our fungi 7 days after incubation at the concentration of 0.5 µl/ml for fraction 4 and 5, and 0.8µl/ml for fraction 3 (table 4). Indeed 3 of the ten fractions of this plant oil are active on the tested fungi but the concentration at which they are active is largely lower than those with the fractions obtain from Callistemon citrinus plant. This observation clearly shown that essential oil from the two plants strongly inhibited the mycelium growth of the tested fungi however Tephrosia vogelii essential oils contain secondary metabolites highly active on the tested fungi compare to Callistemon citrinus. When compare the activity of fraction of those oils with the standards products, Callomil and Manebra, we observe that fraction 4 and 5 of Tephrosia vogelii oil maintain it best activity as it shown inhibition percentage of 100 %, which is similar to the best standard Manebra (table 5). In other to know more about the properties of our oils and fractions, sub culture analysis was done with inoculums from dishes previously used. Results showed that crude extract, fraction 4 and 5 of Tephrosia vogelii have fungicidal properties while only fraction 4 of Callistemon citrinus oil has the same properties. Callistemon citrinus crude oil, its fractions 4 and 5 as well as fraction 3 of Tephrosia vogelii exhibit fungistatic properties against the tested fungi.

Conclusion

In conclusion, the two plants studied have potential to control plant disease due to Fusarium oxysporum lycopersici and could be considered for developing new fungicides. Research should focus on the constituent present in their volatile extract, to purified compound that could be used alone or in association to fight against this pest.

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