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Antifungal Effect of Starch and Biochar Based Essential Oil Formulations in The Preservation of Cowpea (*Vigna Unguiculata* (L). Walp) Seeds

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Abstract

This work focused on the evaluation of antifungal activity of *Thymus vulgaris*, *Cymbopogon citratus* and *Cupressus sempervirens* essential oils formulations based on cassava starch and corncob biochar against fungi associated with cowpea seeds (*Vigna unguiculata* L., Walp) in storage. 200 cowpea seeds were distributed among plastic bottles, 5 g of the starch-based essential oil formulation, for each dose, were incorporated into the seeds. For the formulation of biochar-based essential oils, each piece of biochar soaked with an essential oil dose was inserted into the bottles so as to be in the middle of the seeds. Treated and untreated seeds were kept at laboratory ambient temperature of 25°C ± 2 for 70 days. At intervals of 10, 40, 60 and 70 days of storage, fungal infection rates were assessed by using Agar plate method and Blotter technique. The results showed that 8 fungal species were isolated from cowpea seeds including *Aspergillus flavus*, *A. niger*, *A. parasiticus*, *Cercospora sp*, *Cladosporium sp*, *Fusarium sp*, and *Rhizopus stolonifer* and the percentages of seed infection caused by these different species range from 4 to 51%. Anfifungal effect of starch-based formulations showed that *Thymus vulgaris* essential oil has the highest antifungal activity with 1.66 % and 2% of cowpea seeds infection respectively after 60 and 70 days of storage, followed by *Cupressus sempervirens* and *Cymbopogon citratus* at 7µl/g concentration. For the biochar-based formulation, the essential oil of *C. citratus* at the dose of 7µl/g was the one that showed the highest antifungal activity with 9% of seeds infected after 70 days of storage. Based on these results, starch and biochar can be used for the formulation of essential oils for the preservation of cowpea seeds. *Thymus vulgaris essential* oil is the most effective especially when formulated with starch.

Keywords: Essential Oils; Starch; Biochar; Formulations; Antifungal Activity; Cowpea Seeds; Preservation

Introduction

Vigna unguiculata (L). Walp. (Cowpea) is an important legume of African origin with high protein content. It is cultivated in tropical and subtropical regions like sub-Saharan Africa and widely distributed throughout the world [1-3].

The world's annual production of cowpea is about 7 million tons of dry seeds, of which more than 96% are produced in Africa and 186,000 tons by Cameroon [4]. Legumes in general, and cowpea in particular, make an essential contribution to food security, health and the eradication of global poverty [3, 5], especially in developing countries. These legumes are an important source of proteins, micronutrients and of amino acids. Thus, they can play a key role in fighting protein and energy deficiencies as well as iron deficiency anaemia, one of the most important micronutrient deficiencies in Africa [3]. Many smallholder famers widely value cowpea and other pulses as dual use; legumes used as food or income crops; and for animal feed. However, post-harvest losses due to poor storage are a great cause for concern in the valuation of these crops by farmers.

Storage ensures the availability of agricultural products over a long period of time, thus giving farmers the opportunity to delay their sale and to do it at better prices at the right time [6]. Storage of seeds is certainly the most important post-harvest operation but the losses incurred are great. They are often due to an ineffective control of physical and biological factors, which result in proliferation of insects, moisture and associated fungi. Seeds are particularly susceptible to fungal contamination when stored at high ambient temperature and relative humidity [6, 7].

The damage caused by these storage fungi are exhibited not only by a significant alteration of the aesthetic quality, the organoleptic quality and chemical characteristics of food [7], but also by the production of mycotoxins, such as aflatoxins and fumonisins. These mycotoxins can cause acute or chronic food poisoning in humans and animals, such as carcinogenicity, immune toxicity, neurotoxicity, and hepatotoxicity [7]. The control of post-harvest diseases, is generally achieved by the use of synthetic fungicides [2]. In addition to their high cost, there is a strong debate on the safety aspects of chemical preservatives due to their harmful effects on human health and the environment [8, 9]. For these reasons, consumers tend to be suspicious of chemical additives and thus the demand for natural and socially more acceptable food preservatives is growing significantly and represent a major challenge.

Numerous studies have shown the antifungal activity of essential oils of certain aromatic plant species, including *Thymus vulgaris* [10-12], *Cymbopogon citratus* [13-15] and *Cupressus sempervirens* [16,17] against some storage fungi. Although essential oils being a natural products represent an alternative to synthetic fungicides, there are factors that limit their complete adoption. In fact, essential oils have a relatively short persistence in real storage conditions due to their high volatility and the instability of their constituent molecules [18,19]. In order to ensure their antifungal activity, additional doses are needed, but the extraction yield of essential oils is generally very low.

Hence the importance and the need to develop formulations of essential oils that can optimize efficacy at low doses and thus improve their persistence during food storage, while maintaining their antifungal property. This study was aimed at contributing to the improvement of cowpea grains preservation by the biological control of mold growth using essential oil formulations.

Materials and Methods

Plant material and essential oils extraction procedure

Fresh leaves of *Thymus vulgaris, Cymbopogon citratus* and *Cupressus sempervirens* were collected in January 2020 at Dschang locality. They were air dried at laboratory temperature (25°C ±2) for five days. The leaves were hydro-distilled for about 5 hours using a Clevenger apparatus in the Microbiology and Antimicrobial Substances Research Unit of the University of Dschang. Oils recovered were dried over anhydrous sodium sulphate and stored at 4 °C away from light until they were used to avoid alteration [20,21].

Corncob-derived biochars

The corncob-derived biochars were supplied by the Department of Rural Engineering of the Faculty of Agronomy and Agricultural Sciences. They were produced by carbonizing the corncob biomass using a pyrolysis retort performed at a temperature of 300°C for 6 hours [22,23], then sterilized with the autoclave at 121°C for 15 minutes and stored until use. Biochar in particular corncob biochar has the peculiarity of having a relatively high porosity, almost 80% [24]. This property has justified a particular interest in its use, able to retain essential oils in its micro porosity and allow progressive volatility over time.

Starch

Cassava starch (*Manihot esculenta*) was obtained from the Dschang market. It was sterilized with the autoclave at 121°C for 15 minutes and stored until the time of used.

Formulations of essential oils

Starch-based essential oil formulations

For this work, formulations consisted of pre-defining the mass of powder and the concentrations of essential oils to be used. Referring to previous work [25], the mass of 5g of starch powder and the concentrations of 3 µl/g and 7 µl/g of essential oils were defined for the preparation of formulations. Once the formulations were made, they were let to stand for 10 hours to ensure effective diffusion of volatile elements of essential oil.

Biochar-based essential oil formulations

The essential oils used, at the same concentrations as previous formulations, were only those of *Cymbopogon citratus* and *Cupressus sempervirens* due to their availability. The corncorb-derived biochars were cut into pieces of proportionally equal size (1.7 cm long and 1.4 cm wide). Using a P1000 micropipette, each piece was soaked with a concentration of essential oil and left in airtight plastic bottles for 10 hours before use.

Cowpea seeds treatment

After removing rotten, holed, broken or damaged seeds as well as debris, 200 of these cowpea seeds were distributed among plastic bottles. With regard to the starch-based essential oil formulation, 5 g of this formulation, for each dose, were incorporated into the seeds. Each bottle was then stirred to ensure good contact between the powder and the seeds. As for the formulation of biochar-based essential oils, only the essential oils *Cymbopogon citratus* and *Cupressus sempervirens* were used (due to their availability). Each piece of biochar soaked with an essential oil dose was inserted into the bottles so as to be in the middle of the seeds.

Positive control consisted of cowpea seeds coated with deltamethrin, a synthetic insecticide indicated for the protection of stored seeds, at the manufacturer's dose (2g/kg). The negative control was made up of untreated cowpea seeds. The experiment was replicated three (03) times.

Effect of essential oils formulations

The treated and untreated seeds were kept at laboratory ambient temperature of 25 ± 2 °C for 70 days. At intervals of 10, 40, 60 and 70 days of storage, fungal infection rates were assessed by using Agar plate method (by using Potato Dextrose Agar) and also Blotter technique as described in a previous study [26] with some modifications.

Health status of seeds

To identify the storage fungi associated with cowpea seeds for treated and untreated samples, surface-sterilized cowpea seeds (200 seeds) were plated out on PDA (Potato Dextrose Agar). After 7-10 days incubation at 25 ± 2°C and daily observation, fungal colonies formed around the seeds were collected and purified to identify them according to standard keys of fungi identification as described in other studies [27, 28, 29].

Statistical analysis

Data collected, which are fungal infection rates, were subjected to the separation of means by Duncan's test at the 5% probability threshold through the SPSS (Statistical Package for Social Science) software version 26.0.

Results and discussion

Fungi associated with cowpea seeds and percentage of infection

Table 1 shows the fungal species isolated from cowpea seeds and the infection percentage of each. From these results of health status of cowpea seeds collected in Dschang, they are infected by eight (08) fungal species of interest. These are: *Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus parasiticus, Cercospora sp, Cladosporium sp, Fusarium sp and Rhizopus stolonifer.* The percentages of seed infection caused by these different species range from 4 to 51%.

Antifungal activity of different essential oils formulations

The results of the *in vivo* evaluation of the antifungal efficacy of starch and biochar-based formulations of essential oils of *C. citratus, C. sempervirens* and *T. vulgaris* are presented in Tables 2 and 3. These results show that all formulations have an inhibitory effect on the fungal infection of cowpea seeds. This inhibition varies depending on the type of essential oil, its doses and the storage period.

Table 1: Infection percentage of cowpea seeds before treatment with essential oils by isolated fungi

Fungi species	(%) Fungal infection percentage
Aspergillus flavus	51.00
Aspergillus fumigatus	4.00
Aspergillus niger	10.00
Aspergillus parasiticus	44.00
Cercospora sp	39.00
Cladosporium sp	12.50
Fusarium sp	32.50
Rhizopus stolonifer	19.00

T- : Negative control; T+: Positive control. Means in each column affected with the same letter do not differ significantly at P >0.05 according to Duncan test.

Table 2: Fungal infection rates of cowpea seeds treated with starch-based essential oil formulations with respect to storage time.

Doses (µl/g)		(%) Seed infection percentage		
	days 10	days 40	days 60	days 70
		Starch + *C. citratus*		
3.00	95.50^{c}	37.00^{bcd}	18.00^{b}	23.50^{a}
7.00	95.00^{c}	42.00^{cd}	16.00^{ab}	23.00^{ab}
		Starch + *C. sempervirens*		
3.00	96.50^{c}	28.00^{abc}	43.00^{c}	24.00^{ab}
7.00	94.50^{c}	18.50^{a}	11.50^{ab}	16.50^{acd}
		Starch + *T. vulgaris*		
3.00	87.66^{b}	29.00^{abc}	9.50^{ab}	14.50^{cd}
7.00	94.50^{c}	23.50^{ab}	1.66^{a}	2.00^{e}
		Control		
$^{-}$T	62.50^{a}	62.50^{d}	62.50^{d}	62.50^{f}
$^{+}$T	85.50^{b}	85.50^{e}	85.50^{e}	85.50^{g}

T- : Negative control; T+: Positive control. Means in each column affected with the same letter do not differ significantly at P >0.05 according to Duncan's test.

Table 3: Fungal infection rates of cowpea seeds treated with biochar-based essential oil formulations, depending on storage period.

Doses (µl/g)	Seed infection percentage (%)			
	10 days	40 days	60 days	70 days
		Biochar + *C. citratus*		
3.00	97.50^{c}	45.33^{d}	10.50^{ab}	19.50^{abc}
7.00	97.00^{c}	27.00^{ab}	12.16^{ab}	9.25^{de}
		Biochar + *C. sempervirens*		
3.00	99.50^{c}	51.16^{de}	12.00^{ab}	27.66^{b}
7.00	100^{c}	44.16^{d}	12.66^{ab}	25.16^{b}
		Contrôle		
T $^{-}$	62.50^{a}	62.50^{e}	62.50^{d}	62.50^{f}
T^{+}	85.50^{b}	85.50^{f}	85.50^{e}	85.50^{g}

Antifungal activity of starch-based essential oil formulations

The fungal infection percentages of cowpea seeds treated with starch-based essential oil formulations are shown in Table 2. Based on these results, inhibitory effects of formulations were significantly influenced by storage period, applied dose and the type of essential oil. The percentage of fungal infection of treated seeds with essential oils decreased throughout the storage period.

After 10 days of storage, the infection percentage of treated seeds were high and ranged from 87.66% to 96.50%. These average values are significantly higher ($p<0.05$) than those of positive and negative control (85.50% and 62.50%), respectively.

After 40 days of preservation, the infection percentages of treated seeds were lower than those of control and varied between 18% and 42%. These values are significantly lower ($p<0.05$) than those of the control as well as those obtained after 10 days of preservation.

After 60 days of preservation, the infection percentages of treated seeds that range from 1.66% to 43%, were significantly ($p<0.05$) lower than those of the controls, as well as those obtained after 40 days of preservation. These results show that the antifungal activity of the different formulations depends on the type of essential oil and its dose. To this end, the starch + *Thymus vulgaris* formulation was the one with the highest antifungal activity with fungal infection rates of 1.66% and 2.00% at the concentration of 7 µl /g after 60 and 70 days of preservation respectively, followed by starch + *Cupressus sempervirens* (11.50%) and starch + *Cymbopogon citratus* (16%) 7 µl /g after 60 days of preservation.

Antifungal activity of different biochar-based essential oil formulations

Table 3 shows the percentages of fungal infection of cowpea seeds treated with biochar-based formulations of *Cymbopogon citratus* and *Cupressus sempervirens* essential oils. It appears that after 10 days of treatment, the percentages of fungal infection of seeds range from 97 to 100%, these average values are statistically superior to those of negative and positive controls 62.50 and 85.50% respectively. After 60 days of preservation, the infection percentages of treated seeds that ranged from 10% to 12% were significantly lower ($p<0.05$) than the control, as well as those obtained after 40 days of preservation. It was also noted that these percentages of fungal infection observed after 60 days were also lower than those recorded after 70 days of preservation (9 to 27%). Lowest fungal seed infection percentages were obtained after 60 days of preservation and there was no significant difference between applied doses. However, after 70 days of preservation, the biohar + *C. citratus* formulation showed a better antifungal activity than that observed at 60 days at 7 µl /g, with a seed infection percentage of 9% which is better than that observed at 60 days at 7 µl /g (12%).

Effect of substrate type on the effectiveness of formulated essential oils

Figure 1 presents the effect of substrate type on antifungal activity of *Cymbopogon citratus* essential oil formulations at 3µl /g dose. It appears clearly that the influence of substrate type on seed fungal infection percentages is not statistically signif-

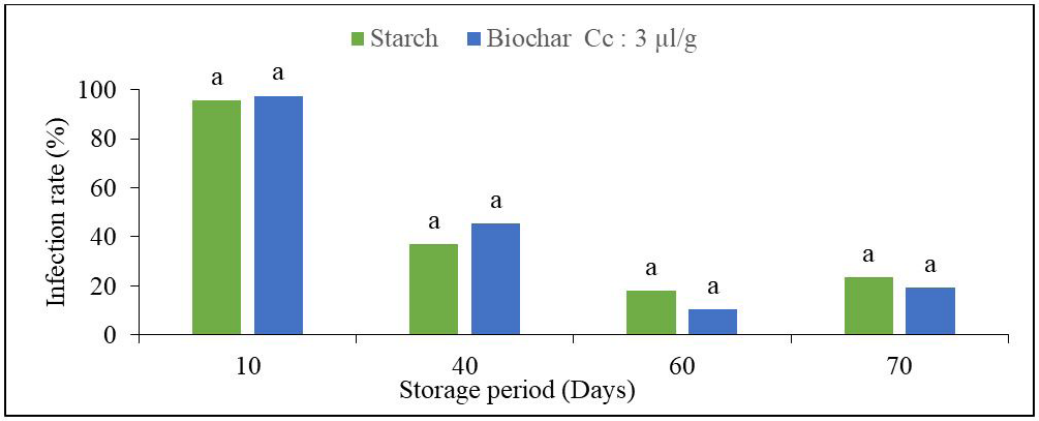

Figure 1: Effect of *Cymbopogon citratus* essential oil formulations at the concentration of 3 µl /g depending on the substrate and storage period. Histograms with the same letter do not differ significantly at the 5% threshold (Duncan's test)

icant ($p>0.05$) at 3µl/g dose. Although at 60 days of preservation where the lowest percentages of fungal infection were observed (Figure 1), starch-based essential oil formulations were more active, but without significant difference with biochar-based formulations.

Figure 2 presents the effect of substrate type on antifungal activity of *Cymbopogon citratus* essential oil formulations at the dose of 7 µl/g. A significant difference ($p<0.05$) between starch and biochar formulations at 40 and 70 days of preservation was noted. The biochar-based formulations had the most pronounced inhibitory effect on fungal infection of the cowpea seeds after 70 days of preservation.

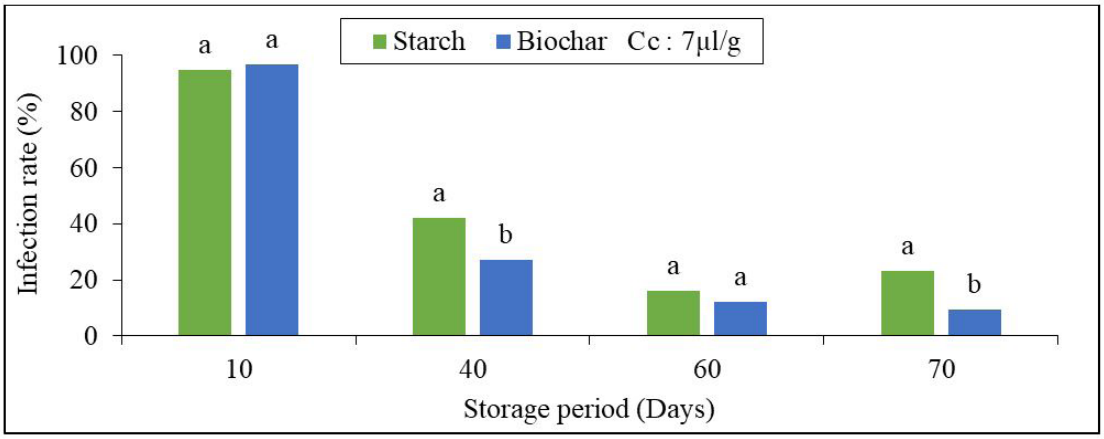

Figure 2: Effect of Cymbopogon citratus essential oil formulations at the concentration of 7µl /g depending on the substrate and storage period. Histograms with the same alphabetical letter do not differ significantly at the 5% threshold (Duncan test)

Discussion

Results from *in vivo* antifungal tests of essential oil formulations of *Cymbopogon citratus, Cupressus sempervirens* and *Thymus vulgaris* based on starch and biochar, against fungi associated with cowpea seeds showed a significant decrease in the rate of seeds infection by the fungi compared to those of untreated seeds and positive controls.

In the case of starch-based formulations, *Thymus vulgaris* essential oil had the highest antifungal activity followed by *Cupressus sempervirens* and *Cymbopogon citratus* at 7µl/g concentration.

As for the biochar-based formulation, the essential oil of *C. citratus* at the dose of 7µl/g was the one that showed the highest antifungal activity.

Fungal infection inhibition by these formulations may be justified by the fact that essential oils used contain active compounds that inhibited the growth of fungi or eventually induce stimulation of the host plant's defense mechanisms against plant pathogenic fungi [30].

Indeed, numerous studies on the chemical composition and antimicrobial properties of essential oils have shown that they are made up of biologically active compounds such as terpenes and terpenoids, particularly monoterpenes and sesquiterpenes with their hydrocarbon and oxygenated derivatives [31, 32]. These studies have reported the fungicide effects of oxygenated compounds and monoterpene hydrocarbons against a diverse group of plant pathogenic fungi [11, 30, 33].

Previous studies [34, 10], have shown that the essential oil of *Thymus* spp is mainly composed of oxygenated monoterpenes such as carvacrol, thymol, linalool, geraniol, and p-cymen. These findings justify the results obtained, and corroborate with the work of [35], which demonstrated that in *in vitro* conditions, *Thymus vulgaris* essential oil significantly inhibited the growth of *Aspergillus flavus*, *A. niger*, *Fusarium oxysporium*, *F. equiseti*, *Penicillium chrysogenum* and *Rhizopus sp* associated with cowpea seeds in storage. Likewise, in *in vivo* conditions, thyme significantly reduced the total incidence of these fungi on naturally infected seeds.

On the other hand, it has been shown that the essential oil of lemongrass has citral aldehyde, β-Citral, geraniol, cis-Verbenol, acetal diethyl citral and nerol as majority compounds, which are oxygenated monoterpenes, as well as myrcene and α-pinene,

which are hydrocarbon monoterpenes [36, 15]. Others workers [37, 38] explained that citral is the chemical component responsible for the antifungal properties of lemongrass essential oil. The work of [39] showed complete inhibition of mycelia growth of *A. parasiticus* by citral. Many other investigations have been conducted on the chemical composition of the essential oils of sempervirens species [16,17, 40-42]. According to these studies, the majority compounds of this oil are monoterpene hydrocarbons, among which α-pinene, δ-3-carène, myrcene and limonene. They also note the presence of oxygenated monoterpenes in very small proportion. Its higher composition of monoterpene hydrocarbons explains why it has the lowest inhibitory activity against fungi associated with cowpea seeds. The work of [16] showed that *C. sempervirens* essential oil moderately reduced the growth of *A. flavus* and *F. oxysporum*. In contrast, the oil was not significantly active against *Rhizopus stolonifer* compared to the positive control (amphotericin B).

To our knowledge, previous work on the formulation of essential oils based on starch and biochar in order to evaluate their biological activity remains unknown. However, we think that the capacities of absorption of these two types of substrates would have an impact on the effectiveness of essential oils in situation of conservation of the cowpea seeds.

Conclusion

The important reduction of fungal development in cowpea grains during storage by these starch and biochar-based essential oil formulations were obtained. The application of starch-based formulations revealed that *Thymus vulgaris* essential oil almost completely inhibited fungi associated with cowpea seeds up to 70 days of storage at 7 µl/g. This was followed by the biochar-based formulation of *Cymbopogon citratus* essential oil with had an acceptable inhibitory effect after 70 days of preservation. In light of these results, starch and biochar can be used for the formulation of essential oils for the preservation of cowpea seeds. *Thymus vulgaris essential* oil was the most effective especially when formulated with starch. It would be interesting to continue this work in order to provide information on the persistence of these formulations.

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