__Review Article__

Antifungal activity of essential oil formulations based on starch and biochar in the postharvest conservation of peanut (*Arachis hypogaea* L.) seeds

Abstract

Poor storage conditions of foodstuffs are responsible for their deterioration by many microorganismspecies with huge quantitative and qualitative losses. The antifungal efficacy of essential oil formulations of some plants based on cassava starch and corn-cob biochar against fungi associated with peanut seeds (*Arachis hypogaea*L.) during storage was evaluated. 200 peanut seeds were dispersed in plastic bottles; 5 g of essential oil formulation starch-based (3 and 7 µl/g)were sprinkled on the seeds. For biochar-based essential oils, pieces of biochar were placed in the middle of the seedsin the bottles. Treated and untreated seeds were stored for 70 days in laboratory conditions. Infection rates of fungal were estimated at 10, 20, 40, 60 and 70 days of storage. From the results obtained, five fungal genera were isolated from peanut seeds, including 4 species of*Aspergillus*, *Cercospora*sp, *Fusarium*sp, *Phomopsis*sp and *Rhizopus stolonifer*, with seed infection percentages ranging from 4 to 62%. The antifungal activity of starch-based formulations revealed that at a concentration of 3 µl/g,*Cymbopogon citratus*essential oil has the highest antifungal activity, with 13% infection of peanut seeds after 70 days of storage. After 70 days of storage, at a dose of 7 µl/g, *C.sempervirens* essential oil had the best antifungal efficacy with 23.50% of infected seeds for the biochar-based formulation. Based on these results, starch and biochar can be used to formulate essential oils for the conservation of peanut seeds. Moreover, *Cymbopogon citratus* essential oil formulated with starch is the most effective.

Keywords: Essential oils; Starch; Biochar;Formulations; Antifungal activity; Peanut seeds; Conservation.

Introduction

Peanut (*Arachis hypogaea* L.) is one of the most important cultivated legumes in sub-Saharan Africa [1-2](Snapp *et al*., 2018; Kpatinvoh *et al*., 2017). Global annual groundnut production is estimated at 47 million tonnes of dry pods, of which nearly 29% is produced in Africa and 594,000 tonnes from Cameroon[3](FAOSTAT, 2018).Pulse in general and groundnuts in particular are essential contributors to global food security, health and poverty eradication [1] (Snapp *et al*., 2018),especially in developing countries. These legumes are an important source of essential protein, micronutrients and amino acids[4] (Asif *et al*., 2013). They can thus contribute to the fight against iron deficiency anemia, one of the most important micronutrient deficiencies observed in Africa and against protein-energy deficiency[1](Snapp *et al*., 2018).

Poor storage conditions are sometimes observed in rural areas. In fact, high seed moisture content (insufficient drying), inappropriate relative humidity and temperature of storage structures, thus promoting mould growth[4-5](Nadjet *et al*., 2016; Taruvinga *et al*., 2014). The damage caused by these stock moulds is manifested not only by a significant alteration of the aesthetic quality and organoleptic and chemical characteristics of the foodstuffs[4](Nadjet *et al*., 2016),but also by the production of mycotoxins such as aflatoxins. The latter are mainly producedby molds of the genus *Aspergillus*, recognized as the major contaminants of peanuts[6] (Torres *et al*., 2014). Due to their varied toxic effects, mycotoxins can cause acute or chronic poisoning in humans and animals, sometimes fatal such as carcinogenicity, immune toxicity, neurotoxicity, and hepatotoxicity[7](Agriopoulou *et al*., 2020).It has been noticed that the many hazards caused by moulds on agricultural products generally leadto their removal during sorting for market, resulting in a worldwide loss of food productionestimated at between 5 and 10%[8] (Yiannikouris and Jouany, 2002).

Nowadays, to reduce the incidence of mould in stored commodities, chemical control through the use of synthetic fungicides seems to be the most effective and widespread control method in the world with a substantial increase in agricultural productivity[9-2] (Aoudou *et al.*, 2010; Kpatinvoh *et al.*, 2017). Nevertheless, synthetic pesticides are currently the subject of global concern since their adverse effects on human healthand the environment have been demonstrated. Indeed, the application of these synthetic chemicals at high concentrations for post-harvest control increases the risk of toxic residues infoodstuffs[10-11](Adjou *et al*., 2013; Rosenbaum *et al*., 2015) and the risk of developing resistant fungal strains[12](Rapp *et al*., 2004).The toxic effects of chemical pesticides in human being are carcinogenic, immunosuppressive, mutagenic, and neurotoxic[13](Mostafalou and Abdollahi, 2016). [4]Nadjet *et al*. (2016)had shown that mycotoxin toxicity could be increased by concomitant human exposure to synthetic pesticides, since the latterinhibit many enzymes, particularly those capable of detoxifying certain mycotoxins.

In view of those problems associated with chemical pesticides, the use of natural products of plant origin for the preservation of foodstuffs is experiencing significant growth and constitutes a major challenge to be met. Essential oils are a new category of biodegradable plant protection products that have demonstrated their antifungal properties, with the particularity of presentingno toxicity to humans[14-15](Abd-Alla and Haggag, 2013; Zhu *et al.*, 2016). Many previous studies on essential oils antifungal activity of *Thymus vulgaris*[16-17, 18](Kritzinger *et al*., 2002; De Vincenzi *et al*., 2004; Vitoratos*et al*., 2013), *Cymbopogoncitratus*[19-20, 21] (Yousef, 2013; Aoudou *et al*., 2017; Premathilake *et al*., 2018) and *Cupressus sempervirens*[22-23](Mazari *et al*., 2010; Ismail *et al*., 2013)have been demonstratedagainstsome stock fungi such as *Aspergillus niger*, *A. flavus*, *Penicillium sp*.*Alternaria alternata*, *Fusarium tricinctum*, *Phomopsis* and *Rhizopus stolonifer*. However, although essential oils do not pose any danger to human health (without overdoses) andenvironment,some factors limited their full adoption. Indeed, essential oils have a short persistence in real storage conditions due to their high volatility and the instability of their constituent molecules[24-25] (Hsieh *et al*., 2006 ; Baptiste Hzounda Fokou *et al*., 2020). In order toensure their antifungal activity, additional doses are necessary but the extraction yield of essential oils is generally very low. Hence the importance and necessity of developing formulations of essential oils capable of optimizing the effectiveness of low doses of these essential oils as well as improving their persistence during food storage, while maintaining their antifungal properties. With this in mind, the objective of this study is to contribute to improve the conservation of peanuts, by fighting against the development of mold using essential oil formulations.

Materials and methods

Plant material and procedure for extracting essential oils

In January 2020, fresh leaves of *Thymus vulgaris*, *Cymbopogon citratus* and *Cupressus sempervirens* werecollected in the locality of Dschang and then air-dried for five days at room temperature (25°C ± 2). The leaves were hydrodistilled forabout 5 hours at theResearch Center for Microbiology and Ontibral Substances of the University of Dschang using a Clevenger-type apparatus. Following the recommendations of [26-27]Sessou*et al*. (2012) and Rguez e*t al*.(2018), the extracted oils were dried with anhydrous sodium sulphate and stored at 4°C away from light until used to prevent spoilage.

Starch and biochar made from corn cob

Cassava starch (*Manihot esculenta*) was obtained by the method described by [28] (Maptue *et al*., 2021).Biochar based on maize cobs was obtained using the methods described by[29]Ioannidou*et al*. (2009), [30] Liu *et al*. (2014) and taken up by[28]Maptue *et al*. (2021). Biochar, in particular corn-cobbiochar, has the particularity of having a high relative porosity ofnearly 80%[31](Djousse *et al*., 2018).This feature has aroused particular interest in its use, as it is able to retain essential oils in itsmicroporosity and allow increasing volatility over time.

Essential oil formulations

Starch-based essential oil formulations *(Cymbopogon citratus*, *Cupressus sempervirens* and *Thymus vulgaris*) were made with reference to the previous work of[32]Camara (2009) and the technique described by[28]Maptue *et al*.(2021). For biochar-based essential oil formulations (*Cymbopogon citratus*, *Cupressus sempervirens*), the technique described by Maptue and collaborators in 2021 was applied. Once the formulations were made, they were left to stand for 10 hours to ensure effective diffusion of the volatile elements of the essential oil.

Treatment of peanut seeds

200 peanut seeds were dispersed in plastic bottles after removing rotten, cracked, or damaged seeds as well as waste. Regarding the starch-based essential oil formulation, 5 g of thisformulation was sprinkled on the seeds for each concentration. Each container was then mixed to ensure excellent contact between the powder and the seeds. Each piece of biochar soaked in a dose of essential oil was placed in the middle of the seeds in the bottles. Deltamethrin, a synthetic pesticide for the protection of stored seeds, used at the manufacturer's recommended dose (2g/kg) was the positive control. Untreated groundnut seeds were used as a negative control. The experiment was performed three (03) times.

Effects of essential oil formulations

For 70 days, treated and untreated seeds were stored at a laboratory room temperature of 25 ± 2°C. Fungal infection rateswere evaluated at 10, 20, 30, 40, 50, 60 and 70 days of storage, using Potato Dextrose Agar and the blotter technique, following the protocol described by[33]ISTA (2023) with some modifications.

Seed health

Disinfected peanut seeds on the surface (200 seeds) were placed on PDA (Potato Dextrose Agar) medium to isolate the associated storage fungus. After 7-10 day incubation at 25 ± 2°C and daily observation, fungal colonies produced around the seeds were collected and purified for identification using conventional fungal identification keys as reported in other studies[34-35, 36](Champion, 1997; Warham *et al*., 1997; Mathur and Kongsdal, 2003).

Statistical analysis

The collected data were subjected to analysis of variance and then separation of the means by the Duncan test at the 5% probability threshold using R software version 4.2.2.

Results and discussion

Fungi associated with peanut seeds and percentage of infection before treatment

Table 1 shows the fungal species isolated from groundnut seeds and the percentage of infection of each. According to this result, groundnutseeds collected in Dschang are infected mainly by eight (08) fungal species of interest. These are:*Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus parasiticus*, *Cercospora*sp, *Fusarium*sp, *Phomopsis*sp and *Rhizopusstolonifer*. The infection ofseeds caused by these differentspecies ranges from 4 to 62%.

Table 1: Percentage of peanut seed infection prior to treatment with essential oils by isolated parasites

	Fungal infection rate (%)
Identified fungi	Peanut
A. flavus	39,00
A. fumigatus	11,50
A. Niger	18,00
A. parasiticus	3,50
Cercosporasp	15,50
Fusarium sp	41,50
Phomopsissp	11,00
Rhizopusstolonifer	22,00

Antifungal activity of different formulations of essential oils

The in *vivo* antifungal efficacy of essential oil formulations of *C.citratus*, *C. sempervirens* and *T. vulgaris* based on starch and biochar is presented in Table 2. These results show that all formulations have an inhibitory effect on fungal infection of peanut seeds. This inhibition varies depending on the type of essential oil, dose, storage period and formulation support.

Table 2: Fungal infection rate of peanuts treated with starch- and biochar-based essential oil formulations as a function of storage period

		Infection rate (%)				
Formulations	**Doses (µl/g)**	**10 days**	**20 days**	**40 days**	**60 days**	**70 days**
Starch +	**3,00**	38.50^{b}	14.50^{a}	22.00^{b}	16.00^{BC}	13.00^{a}
C. citratus	**7,00**	32.50^{b}	24.50^{b}	19.00^{b}	4.00^{a}	24.16^{bc}
Starch +	**3,00**	40.50^{b}	30.00^{bc}	18.66^{b}	15.00^{b}	22.50^{bc}
C. sempervirens	**7,00**	39.00^{b}	41.00^{d}	13.00^{a}	29.00^{d}	28.50^{cd}
Starch +	**3,00**	66.50^{e}	27.00^{b}	11.50^{a}	14.50^{b}	18.50^{ab}
T. vulgaris	**7,00**	18.50^{a}	24.50^{b}	9.00^{a}	14.50^{b}	25.00^{bc}
Biochar +	**3,00**	65.66^{e}	55.66^{e}	44.66^{c}	21.50^{bcd}	34.00^{e}
C. citratus	**7,00**	$54,00^{cd}$	54.66^{e}	$52,00^{d}$	14.66^{b}	57.50^{fg}
Biochar +	**3,00**	40.16^{b}	34.00^{c}	22.50^{b}	24.66^{d}	38.50^{e}
C. sempervirens	**7,00**	52.66^{c}	41.16^{d}	20.16^{b}	23.16^{cd}	23.50^{bc}
Control	**Control -**	62.00^{de}	62.00^{F}	62.00th	62.00^{f}	$62,00^{g}$
	Control +	50.83^{c}	50.83^{e}	50.83^{d}	50.83^{e}	50.83^{fg}

Means in eachcolumnassigned the sameletter do not differsignificantlyaccording to Duncan's test at the probabilitythreshold $P = .05$

Antifungal activity of starch-based essential oil formulations

Figures 1 (A-B) and 2 (A-B) show respectively the percentage of infection as a function of time and the percentage of infection after 70 days of storage of peanut seeds treated with the essential oil formulations of *Cymbopogon citratus*, *Cupressus sempervirens* and *Thymus vulgaris* starch-based.Based on these results, the inhibitory effects of the formulations were significantly influenced by the storage period, the dose applied and the type of essential oil. The percentage of fungal infection of seeds treated with essential oils decreased throughout the storage period. After 10 days of storage, the percentage of infection of treated seeds was high and ranged from 18.50% to 66.50%. After 40 days of storage, the infection percentages of treated seeds were lower than those of positive (58%) and negative (62%) controls; and ranged from 9% to2.2%. These values are significantly lower ($P = .05$) than those of the control as well as those obtained after 10 days of storage. After 60 days of storage, the infection percentages of treated seeds, which range from 14.50% to 29%, are significantly lower ($P = .05$) than those of the controls, but still higher than the infection percentages obtained after 40 days of storage. These results show that the antifungal activity of different formulations depends on the type of essential oil and its dose. For this purpose, the starch formulation + *Cymbopogon citratus* is the one with the highest antifungal activity with a fungal infection rate of 13.00% at theconcentration of 3 µl/g after 70 days of storage, followed respectively by starch + *Thymus vulgaris* (18.50%) and starch + *Cupressus sempervirens*(22%) at a concentration of 3 µl/g after 60 days of storage.

Antifungal activity of biochar-based essential oil formulations

Figures 1 (C-D) and 3 (A-B) show the percentages of fungal infection of peanut seeds treated with essential oil formulations of *Cymbopogon citratus* and *Cupressus sempervirens* based on vegetable charcoal. 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 higher than those of negative and positive controls 62.50% and 85.50% respectively. After 60 days of storage, the percentages of infection of the treated seeds, which vary from 10% to 12%, are significantly lower ($P = .05$) than those of the control, as well as those obtained after 40 days of storage. It was also noted that these percentages of fungal infection observed after 60 days were also lower than those recorded after 70 days of storage (9% to 27%). The lowest percentages of fungal infection of the seeds were obtained after 60 days of storage and there was no significant difference between the doses applied. However, after 70 days of storage, the biochar + *C. citratus* formulation showed better antifungal activity than that observed at 60 days at 7 µl/g, with a 9% seed infection percentage which is better than that observed at 60 days at 7 µl/g (12%).

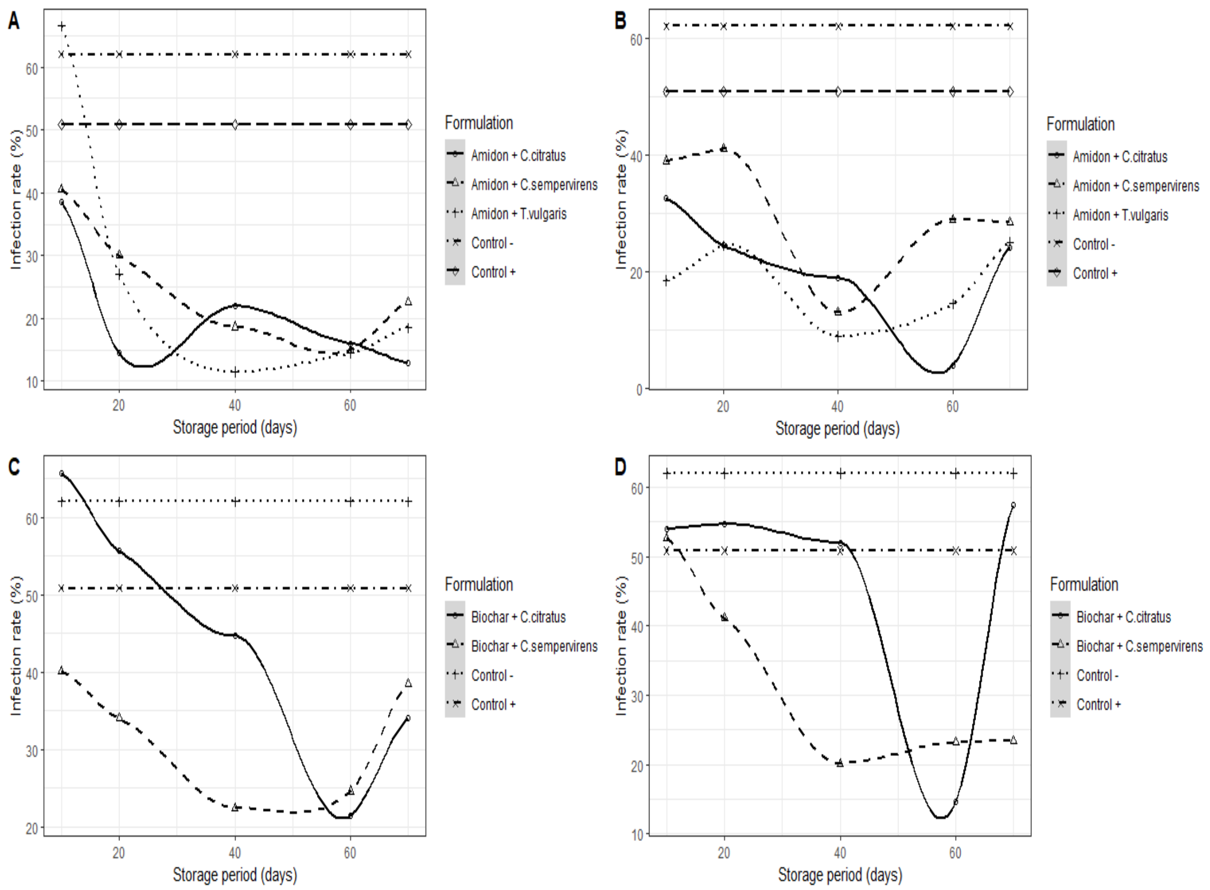

Figure 1: Seed infection rate by substrate type, dose and storage period. A – B: Formulation at a dose of 3 ul/g. C – D: Formulation at a dose of 7 ug/l

Effect of formulation support and dose on the effectiveness of formulated essential oils

Table 2 presents the effect of substrate type on the antifungal activity of essential oil formulations at doses of 3 and 7 µl/ g. It is clear that the influence of formulation support on the percentages of fungal infection of seeds is not statistically significant ($P > .05$) at a dose of 3µl/g. Although at 60 days of storage where the lowest percentages of fungal infection were observed (Figure 1), starch-based essential oil formulations were more active, but with no significant difference from biochar-based formulations.Looking at the effect of formulation support on the antifungal activity of *Cymbopogon citratus essential oil formulations* at a dose of 7 µl/g, asignificant difference ($P = .05$) was observed betweenstarch and biocharformulations at 40 and 70 days of storage. Biochar-based formulations had the most pronounced inhibitory effect on fungal infection of peanut seeds after 70 days of storage.

Discussion

In vivo antifungal tests using the essential oil compositions of *Cymbopogon citratus*, *Cupressus sempervirens* and *Thymus vulgaris* against fungi associated with peanut seeds revealed a substantial decrease in the rate of fungal infection of treated seeds compared to untreated seeds and positive controls. In the case of starch-based formulations, *Thymus vulgaris* essential oil had the highest antifungal activity, followed by formulations of *Cupressus sempervirens* and *Cymbopogon citratus* oils at a concentrationof 7µl/g. In the same vein, forbiochar-based formulations, the essential oil of *C. sempervirens* at the concentration of 7µl/g showed the highest antifungal activity. The ability of these formulations to prevent fungal infections can be explained by the active chemicals contained in essential oils, which can either prevent the development of fungi or stimulate the host plant's defenses such aspathogenic fungi [37](Liu *et al*., 2002).Indeed, numerous studies on the chemical composition and antimicrobial properties of essential oils have shown that they consist of biologically active compounds such as terpenes and terpenoids, especially monoterpenes and sesquiterpenes with theirhydrocarbon and oxygenated derivatives[38-39](Smigielski *et al*., 2018; Tchoumbougnang *et al*., 2009). These studies havereported the antifungal effects of oxygenate and monoterpene hydrocarbons against a diverse group of phytopathogenic fungi[37-18, 40](Liu *et al*., 2002; Vitoratos *et al*., 2013; Regnier e*t al*., 2014).

Previous studies[41-17](Thompson *et al*., 2003; De Vincenzi *et al*., 2004)showed that *Thymus*spp essential oil is mainly composed of oxygenated monoterpenes such as carvacrol, thymol, linalool, geraniol and p-cymen. These conclusions justify the results obtained and corroborate the work of [16]Kritzinger *et al*. (2002), which demonstrated that under 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. Similarly, under 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 lemongrass essential oil has as its majority compounds citral aldehyde, β-Citral, geraniol, cis-Verbenol, acetal diethyl citral and nerol, which are oxygenated monoterpenes, as well as myrcene and α-pinene, which arehydrocarbon monoterpenes[42-16](Shahzadi and Shahzadi, 2017; Premathilake *et al*., 2018;).Otherauthors[43-44](Paranagama *et al*., 2003; Kakarla and Ganjewala, 2015)explained that citral is the chemical component responsible for the antifungal properties of lemongrass essential oil. The work of[9]Aoudou *et al*. (2010) showed complete inhibition of the growth of*A. parasiticus* mycelia by citral. Much other research has been conducted on the chemical composition of essential oilsof evergreen species[45-46, 47, 22, 23] (Chéraif, 2005; Sacchetti *et al*., 2005; Emami *et al*., 2006; Mazari *et al*., 2010; Ismail *et al*., 2013).According to these studies, the majority compounds of this oil are monoterpene hydrocarbons, including α-pinene, δ-3-carene, myrcene and limonene. They also note the presence of oxygenated monoterpenes in very small proportions. Its higher composition of monoterpene hydrocarbons explains why it has the weakest inhibitory activity against fungi associated with peanut seeds. According to [22]Mazari *et al.* (2010),*C. sempervirens*essential oil moderately inhibited the growth *of A. flavus* and *F. oxysporum*. However, compared to the positive control (amphotericin B),the oil was not significantly active against *Rhizopus stolonifer*.

Conclusion

Essential oil formulations based on starch and biochar have significantly reduced fungal development in peanut grains during storage. Application of starch-based formulations revealed that *Thymus vulgaris* essential oil almost completely inhibited fungi associated with peanut seeds for up to 60daysof storage at 7 µl/g. The biochar-based formulation of *Cymbopogon sempervirens* essential oil then had an acceptable effect and inhibitor after 60 days of storage. Based on these results, starch and biochar canbe used in the formulation of essential oils for the preservation of peanut seeds. *Thymus vulgaris*essential oil has proven to be the most effective, especially when formulatedwith starch. It would be fascinating to continue this research to learn more about the durability of these formulations.

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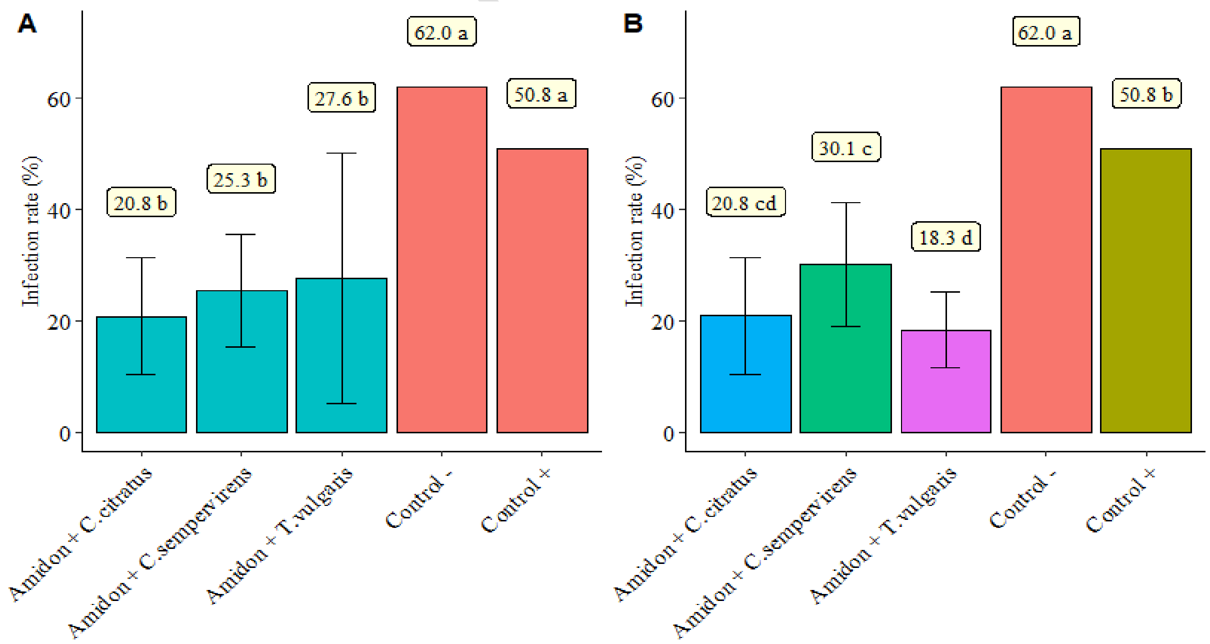

Figure 2: Fungal infection rate of treated peanut seeds based on starch-based essential oil formulations and dose after 70 days of storage. A: Starch formulation at a dose of 3 ug/l. B: Starch formulation at a dose of 7 ug/l.

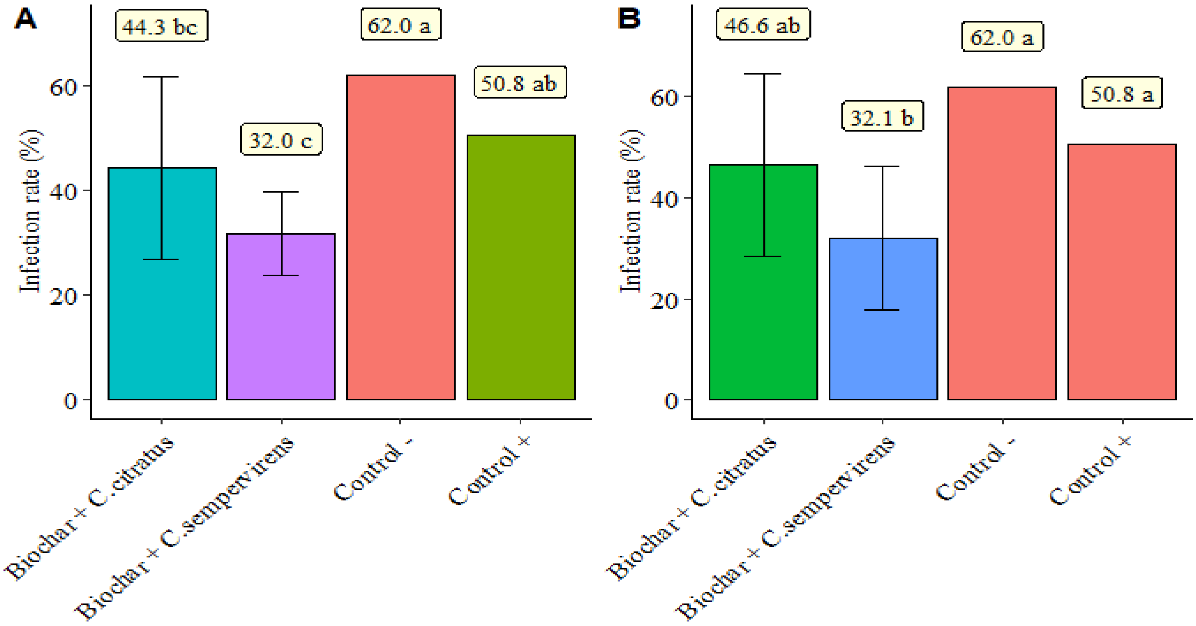

Figure 3: Fungal infection rate of treated peanut seeds based on biochar-based essential oil formulations and dose after 70 days of storage. A: Biochar formulation at a dose of 3 ug/l. B: Biochar formulation at a dose of 7 ug/l.