ESSENTIAL OIL OF *OCIMUM GRATISSIUMUM* AS SEED TREATMENT AGAINST SEED-BONE FUNGI OF SOYBEAN (*Glycine max* L)

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ABSTRACT

Antifungal activity of essential oil of *Ocimum gratissimum* against seed-borne fungi, the possible effect on seed germination and seedlings vigour were investigated. Five soybean seeds cultivars, naturally infected and collected from IRAD of Dschang and Foumbot, West Cameroon, were used. Soybean seeds were distributed among storage plastic, treated with essential oil (0 ; 2 ; 4 and 6 mg/mL) and stored for 40 days in storage conditions. Seed germination percentage, fungal seed infection and seedlings vigour were evaluated each 10 days of storage. Soybean seed treatment with essential oil of *Ocimim gratissimum* significantly reduce seed infection by fungi and also slightly improves soybean seed germination capacity for some cultivars. Non-significant reduction of germination level for ENGOPA 316 and HOULA 1 varieties treated with essential oil were observed. Soybean seedlings obtained from seeds treated with essential oil of *O. gratissimum* were more vigorous than those of untreated seeds. Essential oil of *O. gratissimum* for seed treatments may become a bio-fungicide for soybean protection.

Keywords: Soybean, Seeds, *Ocimum gratissimum*, essential oil, antifungal activity, seedlings vigour

1. INTRODUCTION

Soybean is a primary source of vegetable oil, protein concentrates and starch (Anonymous, 2013, Venugopal, *et al.* 2015). It is an excellent source of major nutrients, about 40% of dry matter is protein and 20% fat (CaldWell, 1973). Due to these nutritional qualities, it is highly used in human and animals nutrition. In Cameroon, Soybean production is mainly carried out in the north and west regions despite the great potential of the country. However, its production is very low in Cameroon because of some constraints such as foliar diseases in which the most recent is the rust, the attacks of pests, and lack of access to improved seeds of good quality (IRAD, 2013).

Seed pathogens have various methods of infection. Some pathogens are soilborne, such as *Pythium*, and are present in the soil. Seed pathogens can be seedborne, seed transmitted, or both. Seedborne pathogens are found on the surface or inside the seed (McGee, 1994). The environmental conditions that promote seed germination usually are favourable for pathogens growth and seed infection. Soybean seeds are thus infected with various seed borne microorganisms, including fungi, bacteria and viruses. Many researchers have reported that *Aspergillus flavus*, *A. niger*, *Cercospora kikuchi*, *Macrophomina phaseolina*, *Fusarium oxysporum* are the most associated soybean fungi (Krishnamurthy and Shashikala, 2006, Krishnamurthy, *et al*. 2008; Impullitti and Malvick, 2013, Lakshmeesha, *et al.* 2013 and 2014, Venugopal, *et al.* 2015, Yaouba, *et al.* 2017a). These fungi are responsible for the loss of germination potential, loss of seedling and rot of seed. Disease free quality seed production in soybean is of utmost importance to sustain productivity and maintain the quality of the crop.

The conservation of seeds in Cameroon especially those of soybean requires much attention. Seeds conservations technologies are very specifics because the embryo have to be kept in good condition to prevent decreasing of germination capacity. The purpose of any seed treatment is to improve seed performance in one or more of the following ways: eradicate seedborne pathogens or protect from soil-borne pathogens ; optimize ease of handling and accuracy of planting (reduce gaps in stand or the need for thinning of seedlings, particularly when mechanical planters are used), and improve germination percentages and rates" (Gatch, 2014). Unfortunately, there are very few organic seed treatments that encourage seedling growth and inhibit fungal growth.

Traditionally, chemical treatments are used widely to protect the germinating seedling, during vegetative and reproductive growth and after harvest from pathogenic fungi infection (Aleieri, *et al.* 1984). Currently, the use and expectations of chemical treatments are greatly concerned due to the impact of the chemical to the environment, which can be primary or secondary influences on human or other living organisms (Baruah, *et al*. 1996). So new strategies for fungicide use and disease management must be developed and identified.

The essential oils of some aromatic plants have antifungal properties. When these essential oils are applied to the seed, they eliminate the soilborne and seedborne pathogens. Velluti et al (2004) showed that the essential oils from oregano, clove, cinnamon, lemon grass, and palmarosa oils reduced the levels of *Fusarium verticillioides* infection in maize seeds. These essential oils also reduced the amount of fumonisins produced by the fungi. Essential oil of *Ocimum gratissimim* have shown their efficiency in controlling *Aspergillus flavus, Penicillium expansum, A. ochraceus, P. verrucosum, Listeria monocytogenes, S.aureus, E. coli, S. typhimurium* (Burt, 2004, Nguefack, *et al.* 2012). However, the use of essential oils as seed treatments for controlling seed-borne infection has been investigated on a limited number of crops (Adegoke and Odesola, 1996, Tagne et Nguefack, 2000). The objectives of this study are to evaluate the efficacy of the essential oil of *Ocimum gratissimum* at controlling seedborne pathogen in some soybean varieties and to determine its effect on seedling growth.

2. MATERIALS AND METHODS

2.1 Plant material and extraction

Fresh leaves of *Ocimum gratissimum* have been collected at Dschang locality in West Region of Cameroon at April 2016, and dried naturally at room temperature for five days. Extraction of essential oil was done in Laboratory of Biochemistry of University of Dschang by hydrodistillation process for about 5 hours, using a Clevenger apparatus. Oil recovered in a dark sterile glass was dried over anhydrous sodium sulphate and stored at +4 °C until it was used (Sessou, *et al.* 2012).

2.2 Soybean seeds varieties

Five cultivars of soybean seeds have been used: MAGBA, TGX-1835-10E, HOULA 1, ENGOPA 316, and TGX-1910-14F, collected from IRAD of Foumbot and Dschang. Seeds where collected in store conditions as recommended by IRAD. They were naturally infected after harvesting.

2.3 Seeds treatment

Treatment have been made using the method of Yaouba et al. (2012) modified and adapted to seed storage conditions as defined by IRAD. Therefore, four small bundles of 15g have been made for all varieties and each of them have been treated with one of the three concentrations of essential oil (0 mg/mL, 2 mg/mL, 4 mg/mL, 6 mg/mL) and then stored for 40 days at room (laboratory) temperature (22 ± 2°C). Data were collected each 10 days using Blotter paper and Agar plate methods for identification of seed-borne fungi and to evaluate the effect of essential oil on seedling vigour. Plastic tray were used to evaluate seeds germination capacity and seedling vigour. Vigour index have been calculated using the formula of Varadarajan & Rao (2002) as shown below:

Vigour index (VI) = Percent germination of seed × (Root length + Shoot length).

In both experiment, untreated seeds for each variety and stored at the same periods served as control. Complete randomized design (CRD) was used in the experiments and each treatment was repeated thrice.

2.4 Statistical analysis

Statistical analysis of data of three independent replicate trials were done using R version 3.2.3 and SPSS 21 (IBM). Differences between means were tested using Duncan test à $p< 0.05$, and principal components analysis (PCA) has been carried out in order to show relations between experimental factors.

3. RESULTS AND DISCUSSION

3.1 Prevalence of seed-born fungi of soybean varieties

Fungal species have been identified on all the soybean seeds varieties. Variety TGX-1835-10E was mostly infected by *A. flavus, A. niger, Penicillium* sp, *Cladosporium cladosporioides* with varied percentage. *Fusarium moniliforme, F. solani, F. oxysporum* and *Cercospora kikuchii* were present on all varieties with high infection percentage on the varieties collected from Foumbot locality. *F. oxysporum* was present on ENGOPA 316 (5.4%) and absent on both MAGBA and TGX-1835-10E collected from IRAD of Dschang. *Phomopsis* sp., *Rhizopus stolonifer*, and *Colletotrichum* sp. causative agents of high losses of germination and seedling losses have been identified on the seeds collected from Foumbot. Other fungi like *Botrytis* sp, *Chaetomium* sp, *Cladosporium cladosporioides*, *Melanospora zamiae*, *Mucor* sp, *Nigrospora* sp, *Penicillium* sp, *Peronospora* sp, *Phoma* sp, and *Trichoderma* sp were also isolated with infection percentage variable according to each variety.

3.2 Germination of soybean seeds treated with *Ocimum gratissimum* essential oil

The results of effect of essential oil on germination of soybean seeds after 0 ; 20 and 40 days of storage are illustrated by figures 1, 2 and 3. Before storage, treated and untreated soybean seed were assessed for germination. For untreated seeds, percentages of germination varied from 59.7 (TGX-1910-14F) to 96.7 (TGX-1835-10E). Significant effect of essential oil on the germination rate was observed (**Figure 1**). For this purpose, MAGBA and TGX-1910-14F varieties showed germination percentages of 93.7 (2 mg/mL) and 85 (2 mg/mL) respectively, which are significantly higher than those of their control. There is no significant difference between the germination rate of seeds treated with essential oil and control for other varieties. However HOULA 1 variety presented notorious lower germination at 6 mg/ml. **Figure 2** illustrates the effect of essential oil on germination after 20 days of storage. It is clear from these results that ENGOPA 316 and HOULA 1 varieties showed a significant reduction of germination percentage which fell from 71 to 53 and from 64.5 to 52 percent, respectively, at 6 mg/mL. However TGX-1910-14F variety presented a significant increase of its germination rate compared to the control. After 40 days of storage (**Figure 3**), no significant reductions of germination rates noted for these soybean varieties outside of TGX-1910-14F variety which has presented a significant increase of germination percentage for treated seeds.

3.3 Effect of essential oil on seed-born fungi

Seeds infection percentage after 10; 20 and 40 days of storage are presented in table 1. It is clear that all concentrations of *O. gratissimum* have significantly reduced the infection rate in all soybean varieties compared to the control where the infections percentage ranged from 42 (MAGBA) to 68% (TGX-1910-14F). The reduction of the fungal infection is function of essential oil concentration and soybean variety.

Up to 40 days of storage, the infection percentages recorded on different varieties of soybean seed treated with essential oil of *O. gratissimum* are significantly lower than those of the untreated control. A 10 days, ENGOPA 316 and MAGBA varieties have been more sensitive to the essential oil effect with infection rates of 7 and 5% at 6 mg/mL respectively. A 40 days of storage, all essential oil concentrations remained effective but much more for 4 and 6 mg/mL.

3.4 Effect of essential oil on seedlings vigour

Figures 4, 5 and 6 show the effect of essential oil of *O. gratissimum* on the vigor of tested soybeans seedlings varieties. After seeds treatment with essential oil and before their storage the vigor of soybean seedlings was evaluated. So VI obtained for MAGBA, TGX-1835-10E and HOULA 1 seedlings are not significantly different for those of the control. On other hand, TGX-1910-14F showed a significant decline in its vigor index to 4 mg/mL (470). However VI improved at all concentrations used are registered on the ENGOPA 316 seedlings ranging from 764 to 1189. After 10 days of conservation, the vigor of seedlings from treated seed with essential oil are significantly higher than those of control. ENGOPA 316 presents the highest VI (1966) at 6 mg/mL, MAGBA and HOULA 1 respectively 2638.5 and 2123 at 4 mg/mL concentration. TGX-1910-14F seedlings present best vigor of 1952 at 2 mg/mL. 40 days of storage, there was a decrease of the effect of essential oil on the vigor of seedlings. Thus, seedlings of MAGBA, ENGOPA 316 and TGX-1910-14F varieties have presented their best vigor which are respectively 1780.5, 1615 and 1952 at 2 mg/mL of concentration. Only the HOULA 1 variety has maintained its best strength at 4 mg/mL. There is noted that from the 20th day of storage, the effect of essential oil on the vigour of seedlings of certain varieties has not changed, such is the case for example of the TGX-1910- 14F variety whose seedlings presented the same strengths for 20th to 40th day of analysis.

3.5 Correlations between seeds germination percentage, seeds infection and seedling vigour

This study has assessed the antifungal activity of essential oil (EO) of *Ocimum gratissimum* against seed-borne fungi of some soybean varieties present in West Cameroon and the effect on seedlings vigour. In figure 7 it is noted that vigour index has significant negative correlation with seeds infection percentage. Similarly there is a positive correlation between vigour index and storage time, meaning that vigour index increase with time and can be reduced by the increase of seeds infection rate. In figure 8, it can be also seen that all treatment are negatively correlated to infection percentage except Treatment 1 which the control (0 mg/mL). Therefore all treatment significantly reduced seeds infection percentage except the control (Trt 1). Figure

9 reveals that germination percentage and percentage of rotten seeds depends of soybean varieties.

4. DISCUSSION

The study was conducted to investigate the effect of essential oil of *Ocimum gratissimum* at different concentrations (0 ; 2 ; 4 and 6 mg/mL) on seeds germination, seeds infection and seedling vigour on five soybean varieties. The results showed that the essential oil significantly ($P \leq 0.05$) reduced the incidence of natural soybean seeds infection and slightly improved seed germination of some variety when compared with untreated control seeds.

The essential oil of *O. gratissimum* was found to be most effective against seed-borne fungi. This effect depend on its chemical composition and on the presence of certain compounds which are known for their antifungal activities. The GC analysis of *O. gratissimum* essential oil from Cameroon showed as main constituents, thymol (47.7%), c-terpinene (14.3%) and cymene (8.5) (Ngassoum, *et al*. 2003, Tatsadjieu, *et al*. 2009). Similarily, Sessou et al (2012) showed that *O. gratissimum* essential oil from Benin had as main components thymol (28.1%), c-terpinene (21.30%) and p-cymene (16.5%) and as other minor compounds in significant percent myrcene (7.2%), ý-thujene (5.8%) and limonene (2.5%). Many studies have assessed antifungal activities of essential oil of *Ocimum gratissimum* against different foodborne pathogens. It was reported that volatile oil of *O. gratissimum* had significant antimicrobial effects against both fungi and bacteria (Tagne, *et al*. 1998).

The biological activity of this oil is probably due to its prominent concentration in thymol which is a phenolic compound. Generally, the essential oils possessing the strongest antimicrobial properties against food borne pathogens contains a high percentage of phenolic compounds such as carvacrol, eugenol (2-methoxy-4-(2-propenyl) phenol) and thymol (Mew, *et al*. 1986, Tassou, *et al*. 2000). According to Nguefack et al (2007), *Ocimum gratissimum* essential oil detains good antifungal properties against mycotoxinogenic fungi. An important characteristic of thymol is its hydrophobicity, which enables it to partition in the lipids of the fungal cell membrane, disturbing the structures and rendering it more permeable and leakage of ions and other cell contents can then occur (Mew, *et al.* 1986). According to effect of *O. gratissimum* on seed germination it was observed that at 6 mg/mL, *O. gratissimum* reduce the germination rates of HOULA 1 and ENGOPA 316 varieties especially in the first 20 days of storage. According to Rodrigues, Rodrigues and Reis (1999), a essential oil compound inhibit germination and growth by interfering with cell division, membrane permeability and the activation of enzymes. Moreover it is also noted more or less significant improvements in the level of germination of other varieties. TGX-1910-14F presented a significant increase of its germination rate which rose from 60 (control) to 96 at 6 mg/mL. Tagne et al (2008) repported that the germination of treated maize seeds with the essential oil of *O. gratissimum*, shows important improvements between the treated seed and the untreated control. Nguefack et al (2008) showed that essential oil of *O. gratissimum* increased the germination capacity of the treated rice seeds with 5 to 13%. According to the improvement of germination, the ability of some plant extracts to increase seed germination could be attributed to the suppression of seed borne fungi that could have consider to kill the embryo of the seeds leading to germination failure. Parimelazhagan and Francis (1999) established an increase in germination rates and an improvement in seedling development of rice seeds with leaf extract of *Clerodendrum viscosum*. An improvement of vigour of soybean seedlings was also recorded during this study. This can be illustrated by VI presented by ENGOPA 316; HOULA 1 ; TGX-1910-14F and MABGA which are 1615, 1827, 1952 and 1780 respectively after 40 days of conservation. These index are significantly higher than those of the untreated control. Tagne et al (2008) repported that the seedling vigor from treated maize seeds with the essential oil of *O. gratissimum*, shows important improvements between the treated seed and the untreated control. Treatment of groundnut seeds with 0.05 and 0.15 g/ml concentration of the essential oil of *Callistemon viminalis* for 1hr showed growth and germination improvement and significant decrease of seed mycoflora infecion (Yaouba, *et al*. 2016). In this present study it is well demonstrated that the control effect of essential oil from *O. gratissimum* on the seed-borne fungi of soybean is well demonstrated and that the EO could be used as seed treatments for soybean with the same expected output as obtained with synthetic fungicide.

5. CONCLUSION

Soybean seed treatment with essential oil of *O. gratissimum* significantly reduce seed infection by fungi and also improves soybean seed germination for some varieties. Non-significant reduction of germination level for ENGOPA 316 and HOULA 1 varieties treated with essential oil were observed. Soybean seedlings obtained from seeds treated with essential oil of *O. gratissimum* were more vigorous than those of untreated seeds. Essential oil of *O. gratissimum* for seed treatments may become a bio-fungicide for soybean crop protection. However, further investigations and product developments are needed as well as field experiments.

6. ACKNOWLEDGEMENTS

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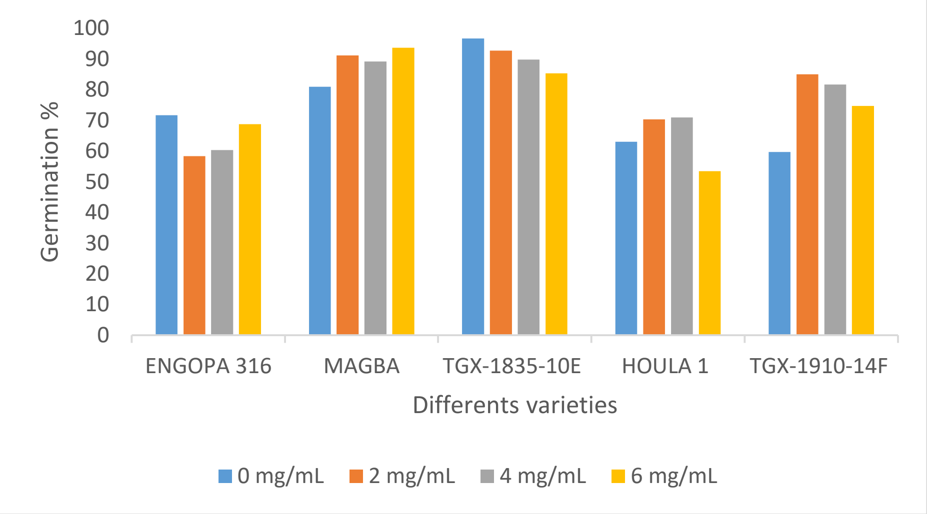

Figure 1 : Germination percentage of soybean varieties before storage

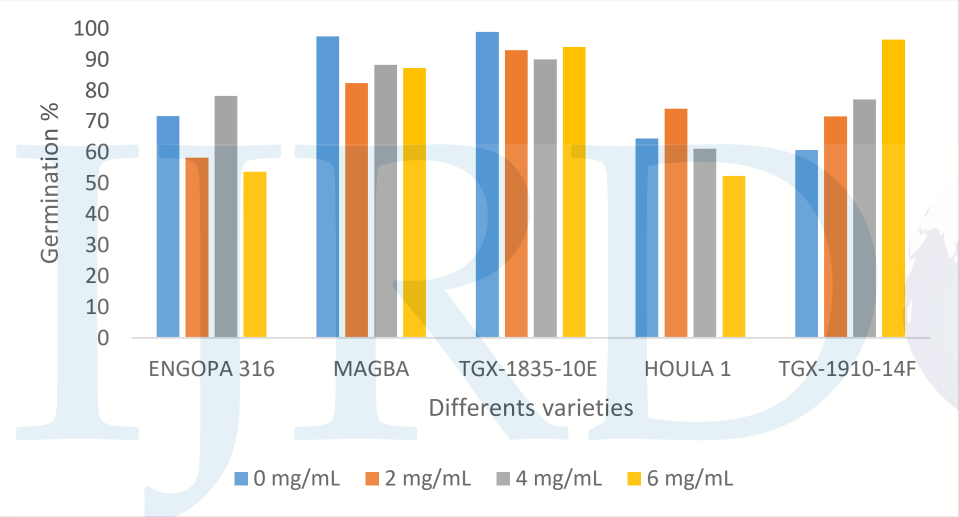

Figure 2 : Germination percentage of soybean varieties at 20 days of storage

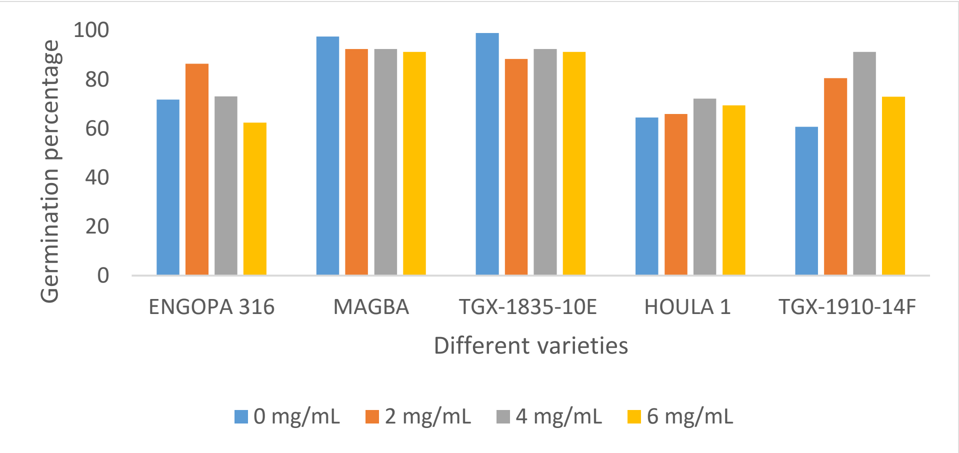

Figure 3 : Germination percentage of soybean varieties at 40 days of storage

Tableau 1 : Fungal seeds infection percentage after 10, 20 and 40 days of storage

Variétés	Concentrations			
	0 mg/mL	2 mg/mL	4 mg/mL	6 mg/mL
		10 days of storage		
ENGOPA 316	53,7 ± 15,3a*	8,9 ± 8,4b	13,6 ± 3,4b	7,1 ± 6,7b
MAGBA	42 ± 19a	4,1± 0,8b	3,7 ± 3,3b	5,3 ± 5b
TGX-1835-10E	67,2 ± 4,9a	6,4 ± 3,4b	13,7 ± 3,3b	12,3 ± 2,5b
HOULA 1	62 ± 30,1a	10,7± 10b	8,8 ± 3,3b	16 ± 15,1b
TGX-1910-14F	67,8 ± 32,3a	20,7 ± 10,1b	24,2 ± 13,4b	11,7 ± 1,6b
		20 days of storage		
ENGOPA 316	53,7 ± 15,3a*	39,4 ± 4,1b	29,3 ± 10b	27 ± 7,5b
MAGBA	42 ± 19a	14,7± 9,2b	16,6 ± 10,9b	10,6 ± 14,2b
TGX-1835-10E	67,2 ± 4,9a	24,1± 0,8b	41,7 ± 1,7b	36 ± 15b
HOULA 1	62 ± 30,1a	45,9± 0,8b	20 ± 0,0b	12,3 ± 2,5b
TGX-1910-14F	67,8 ± 32,3a	10,9 ± 0,8b	16,4 ± 3,4b	35,9 ± 0,85b
		40 days of storage		
ENGOPA 316	53,7 ± 15,3a*	13,6 ± 3,4b	4,7 ± 5b	10 ± 0,0b
MAGBA	42 ± 19a	7,6 ± 2,5b	15,3 ± 5b	8,9 ± 8,4b
TGX-1835-10E	67,2 ± 4,9a	16,7± 3,3b	11,7 ± 1,6bc	7,1 ± 6,7c
HOULA 1	62 ± 30,1a	17,4± 7,5b	4,2 ± 0,8b	7,1 ± 6,7b
TGX-1910-14F	67,8 ± 32,3a	5,3 ± 5b	10,5 ± 4,1b	10,1 ± 0,2b

Values with the same letter on the same line are not significantly different at $P \leq 0.05$ according to Duncan test

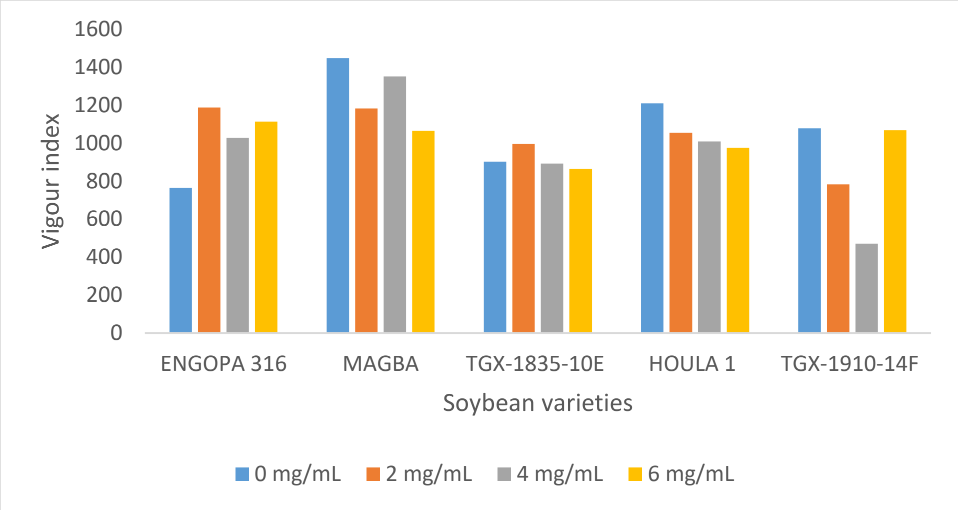

Figure 4 : Seedlings vigour index obtained before storage

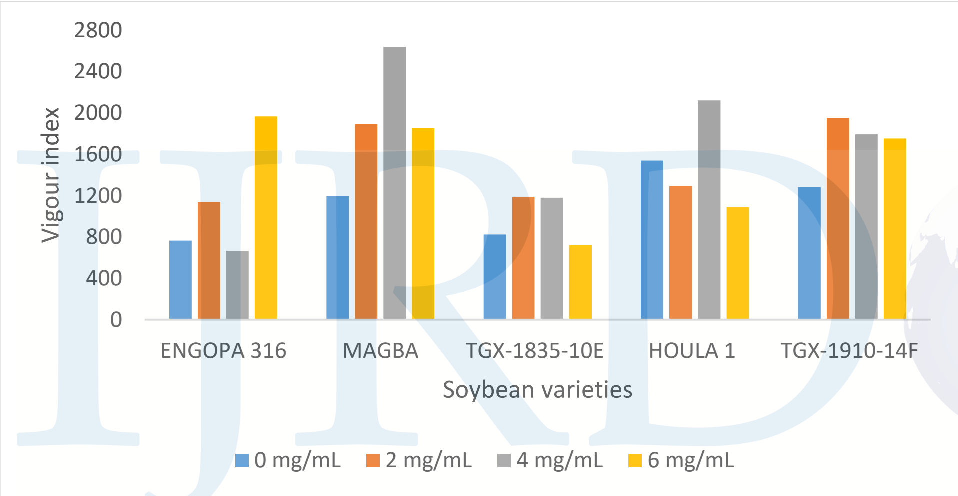

Figure 5 : Vigour index of seedlings at 20 days of storage

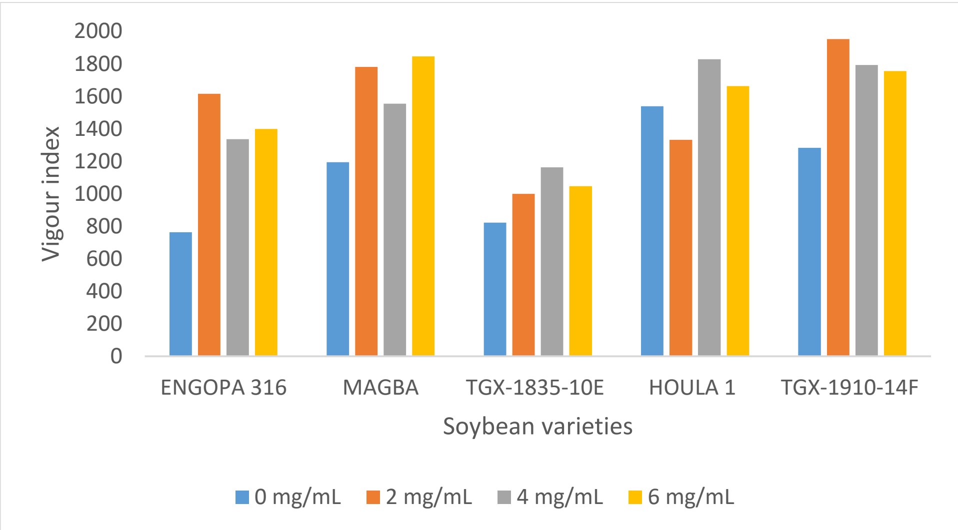

Figure 6 : Vigour index of seedlings after 40 days of storage

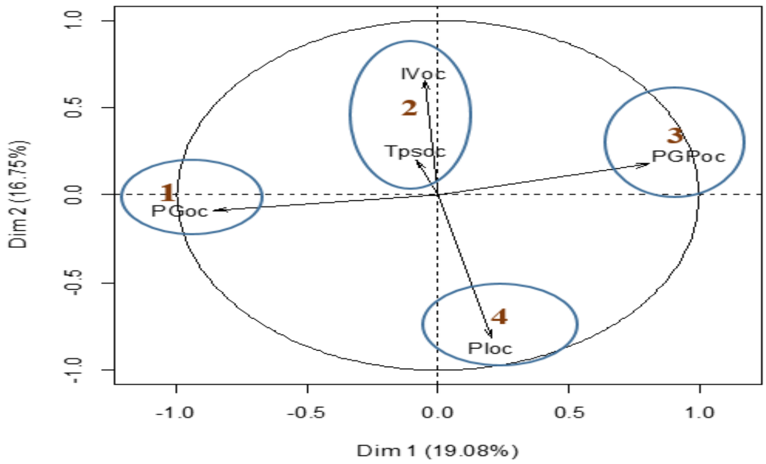

Figure 7: Relation between seedling vigour and seeds infection

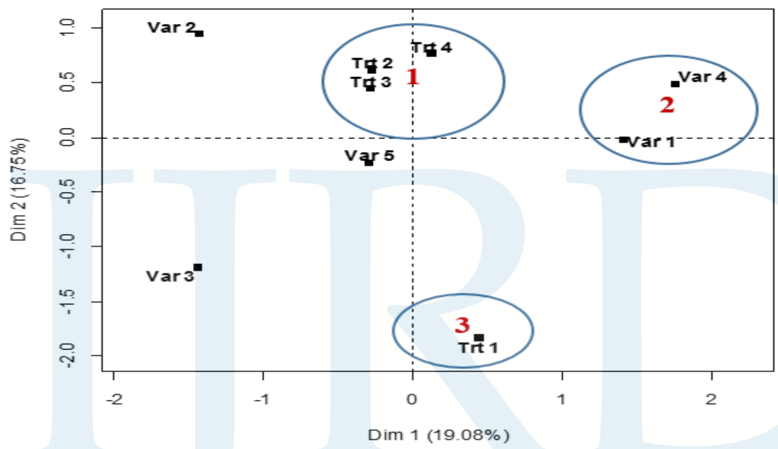

Figure 8: Relation between essential oil and seeds infection

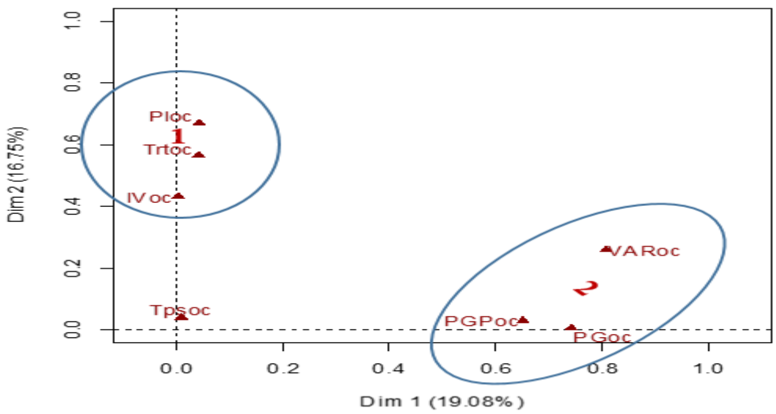

Figure 9: Relation between seed germination and soybean varieties

IVoc : Vigour Index; Tpsoc : Time of storage; PGPoc: Percentage of rotten seeds; PIoc: Percentage of infected seeds; PGoc: Germination Percentage; VARoc: Varieties; Trt 1 : 0 mg/mL; Trt 2 : 2 mg/mL; Trt 3 : 4 mg/mL; Trt 4 : 6 mg/mL; Var1: ENGOPA 316 ; Var2: MAGBA ; Var3:TGX-1835-10E ; Var4 : HOULA1 ; Var5 : TGX-1910-14F