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Microencapsulation of *Lippia turbinata* essential oil and its impact on peanut seed quality preservation

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ABSTRACT

In this study the use of complex coacervation to encapsulate the essential oil (EO) of *Lippia turbinata* (poleo) and its application for controlling fungal pathogens of peanut seeds were evaluated. High percentages of encapsulation of poleo EO were obtained (99.80%). At the end of storage period (78 d), between 3.5 and 63% of poleo EO were released from microcapsules, this action was favoured by the increase of grain a_W. The formulation of poleo EO showed a significant antifungal effect on peanut mycoflora, with reductions between 59 and 77%. Mycological studies showed a prevalence of *Penicillium* and *Aspergillus* lesser extent in peanut seeds throughout the storage period. The formulation caused complete inhibition of peanut seeds germination. In conclusion, the gelatin/gum arabic system was effective in encapsulating poleo EO by allowing its controlled release. Microencapsulated poleo EO maintained the antifungal activity, but produced allelopathic effects on peanut seeds germination.

1. Introduction

Peanut (*Arachis hypogaea* L.) is an economically important crop throughout the world. Argentina is the sixth producer of peanut with 3.0% of overall world production, ranking first in the category of peanut exporters worldwide (USDA, 2015). Córdoba province is responsible for 93% of the national peanut production with a cultivate area of 345,200 ha (BCCBA, 2015). Nevertheless, this crop is susceptible to many pathogens, with most damage caused by fungi. Soil-borne fungal diseases adversely affect peanut health and productivity all over the world growing areas. Depending on severity of field infestation, yield losses due to such soil-borne disease may be as high as 50%. The diseases are caused by seed borne pathogens that can survive in infected peanut seeds (Melouk and Backman, 1995). Therefore, harvested peanut seeds contain fungal mycelia and spores that can result in a significant decrease in seed quality when they are stored. This is an important aspect, considering that in our country producers preserve peanut seeds for the next planting harvested over a period of 4–6 months. During storage, a microorganism's succession is development on the grains, which is determined by physical–chemical (moisture, temperature, pH, levels of O_2, chemical additives and storage time) and biological conditions (interactions with other microorganisms, presence of insects and rodents) that allow transitory or permanent changes in the fungal population (Lacey and Magan, 1991). The most common fungi identified from peanut seeds stored in different storage systems included *Penicillium*, *Aspergillus*, *Eurotium* and *Fusarium* spp. Within *Aspergillus* genus, the section *Flavi* had the greatest mean counts of 1.4×10^4, 9.4×10^2, 5.2×10^2 cfu g^{-1} for big bags, wagon and warehouse, respectively (Passone et al., 2014). Consequently, storage fungal infection can reduce the quality and the seed viability. Seeds contaminated with pathogenic fungi can act also as a source of inoculum for diseases by affecting the crop during its development, or as a vehicle for dispersion through which different pathogens can be introduced in a field or region. Therefore, the quality of the seeds is essential to establish an appropriate stand of plants for the production of peanut crop (Ketring, 1991).

Usually the germination and seed vigor tests are evaluated, but their sanitary quality is generally not considered, assuming sufficient conduct a seed fungicide treatment (March et al., 2003).

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Effective control of root and stem wilt diseases can be reached by supplying a recommended fungicide. Thus, seed treatments with chemical fungicides, such as thiophanate methyl [dimetil-4,4'-(o-fenileno) *bis* (3-tio alofanato)] and metalaxyl [metil D,L-*N*-(2,6 dimetil fenil)-*N*-(2-metioxi-acetil)-alaninato] are frequently used in combined form to protect the seed from pathogenic organisms before sowing. Both agrochemicals are systemic fungicides that play a very important role in plant disease control and they are applied world-wide on numerous crops. The fungicidal activities mechanism of thiophanate metyl (carbamate group) is due to the cholinesterase inhibitory activity, while metalaxyl (derivative of acilanines) causes protein synthesis inhibition (Leroux, 2002). This strategy could contribute to environmental pollution added to the hazard that it normal use represent on human health (Silva and Fay, 2006; Pires et al., 2005). Therefore, there is a considerable interest in finding alternatives to synthetic chemical pesticides for suppression of soil-borne plant pathogens (Haggag, 2007).

The use of natural compounds offers an alternative in replacing synthetic chemicals intended to plant diseases control. Essential oil (EO) of *Lippia turbinata* (poleo) has proven *in vitro* effectiveness for controlling *Aspergillus* on culture medium and on irradiated peanut kernels by contact (Passone et al., 2012a; b) and vapor tests (Passone et al., 2013; Passone and Etcheverry, 2014). However, EO levels quickly decreased when it was applied in the peanut food systems. Microencapsulation technology is one of the most effective methods to date to achieve controlled release of the compounds (Moretti et al., 2004; Hussain and Maji, 2008). This process creates a physical barrier between the core and wall materials protecting sensitive ingredients from the external environment, particularly moisture, pH and oxidation (Nesterenko et al., 2013).

Thus, the aims of this work were: i) to encapsulate poleo oil by complex coacervation method; ii) to evaluate the encapsulation parameters; iii) to analyze the microcapsule application for controlling fungal pathogens in peanut intended for seed; and iv) to study the effect of microencapsulated poleo oil on peanut seed germination.

2. Materials and methods

2.1. Collection and characterization of the essential oil

Dried leaves of *Lippia turbinata* (poleo) were purchased from a local market. The identification was done according to Cantero and Bianco (1986). The plant material was stored at 4 °C after harvest. A portion (100 g) was submitted for 3 h to water-distillation, using an extractor of essential oils (EOs) by steam distillation at laboratory scale (Figmay S.R.L.) (yield 1.02%). The obtained EO was dried over anhydrous sodium sulfate and, after filtration, stored in a sterilized vial at 4 °C. The characterization of poleo EO was performed by gas chromatography mass spectrometer (GC/MS) (Passone and Etcheverry, 2014).

2.2. Preparation of coacervate microcapsules

Poleo EO was used as core material, while gelatin (type A, gel strength 240 bloom) and gum arabic were used as the wall material. The microcapsules were made by complex coacervation adapting the methodology proposed by Girardi et al. (2015).

Twenty five mL of gelatin and gum arabic solutions 5% w/v were prepared at 50 °C in a thermostatic bath (Decalab SRL). pH of gum arabic solution was adjust to 6 with sodium hydroxide 1M (NaOH). Poleo EO (450 μL) was added into the gum arabic solution, forming an emulsion by magnetic stirring (Auto Science, AM-5250B). Then, gelatin solution was added and the mix was stirred at 400 rpm during 10 min at 50 °C. After that, pH was adjusted to 4 with hydrochloric acid 1M (HCl) and the stirring was continued for 10 min. Subsequently, pH was adjusted to 9 with NaOH 1M and stirred for 10 min. After that, the temperature was lowered to 10 °C in an ice bath and 5 mL of formaldehyde was then added to compact the gelatin/gum arabic coating. Crosslinking time was 10 min at room temperature.

Finally, microcapsules were washed twice with distilled water and were stored at −20 °C until the lyophilization step. For lyophilization process, microcapsules were previously frozen at −80 °C during 3 h (L-T8-A-B3-CT, RIFICOR). Then the samples were ground (CT 193 Cyclotec™ Sample Mill) to obtain a fine powder (1000 μm).

2.3. Efficiency of encapsulation

Efficiency estimation was adapted from Kaushik and Roos (2007). Microcapsules (0.5 g) were added to 10 mL of chloroform (HPLC Grade, Sigma Aldrich) in glass flask and shaken in an orbital shaker for 5 min. Powder particles were separated from chloroformic extract by filtration. The test was performed in triplicate.

The amount of EO present on the surface of the microcapsules was estimated from chloroformic extracts by gas chromatography mass spectrometer (GC/MS Clarus 600, Perkin Elmer) equipped with a DB5 column (60 m, 0.25 mm ID, 0.25 μm particle Perkin Elmer). To control the equipment and data acquisition the Turbo Mass program was used. Working conditions were: initial temperature 60 °C (5 min) ramp: 5 °C/min, final temperature 115 °C. The mobile phase used was Helio 5.0 to 49.6 psi. The temperature of the injector and GC transfer line was maintained at 250 to 200 °C, respectively. Ionization was performed in the mass spectrometer vacuum with electron impact ionization energy −70 eV. The injection volume was 1 μl. The chromatogram was obtained in 'scan' mode from m/z = 50 to m/z = 350 (scan time 0.2 s, inter-scan time: 0.1s). The identification of the components of the poleo EO was performed by comparison with spectra libraries NIST MS Search 2.0.

For quantification of the residual oil, an external calibration curve of limonene (MW: 136.23 g/mol; CAS: 5898-27-2) was used, due it was the main component of pure poleo EO. The quantification curve in the range of sample concentrations (0.008232−1.0976 μg/μL of limonene, $R^2 = 0.9626$) was performed. Each concentration level of standard solution was analyzed by GC/MS in triplicate. Quantification was performed by reporting the measured integration areas in the calibration equation of the corresponding standards. The detection (LOD) and quantification (LOQ) limits of the analytical method for limonene 0.02 and 0.82 ng/g were 0.05 and 0.30 ng/g.

The encapsulated poleo oil was determined as the difference between the theoretical initial level and values estimated on the microcapsule surface. Efficiency encapsulation was expressed as the percentage of poleo oil encapsulated with respect to the initial amount.

2.4. Release property of poleo EO microcapsules

Release of microencapsulated poleo EO was evaluated *in vitro*. To characterize the formulation stability, 0.1 g of microcapsules were placed in flaks, which were then sealed and incubated at 25 and 4 °C. To evaluate the release of microencapsulated poleo oil on the substrate, peanut kernels were sterilized twice by autoclave at 120 °C for 20 min. Then water activity (a_W) was adjusted by aseptically adding sterile distilled water according to the calibration curve previously made. Sealed containers were stored at 4 °C for 48 h with periodic hand shaking during this time. Water activity values were confirmed with an AquaLab Series 3 (Series 4, TE, USA)

with an accuracy ± 0.003. Finally, 0.1 g of microcapsules was placed into a flask containing 5 g of sterile peanuts conditioned at 0.65, 0.75, 0.85 and 0.95 a_W. The flask was sealed and incubated at 25 ± 2 °C for 78 d. For both assays the extraction of poleo EO residues were performed at different times (5, 23 and 78 d).

Anhydrous ethanol (10 mL) was added to each flask and stirred for 5 min in an orbital shaker. Powder particles were separated by filtration. Poleo EO residues present in the ethanol extract were quantified using a Folin-Ciocalteau technique for the determination of total polyphenols (Schlesier et al., 2002; Deladino et al., 2008). For this, 2 mL of Na_2CO_3 (2% w/v) were mixed with 200 μL of the ethanol extract and 200 μL of Folin-Ciocalteau reagent. After 30 min of incubation, sample absorbances were measured at 725 nm in a spectrophotometer (Spectrum SP 2100UV). The same procedure was also applied to the standard solutions of gallic acid (0–1000 μg/0.1 mL), obtaining the following standard curve: Absorbance = 0.0012*x(μg) + 0.00256. Total polyphenols of poleo EO were determined as gallic acid equivalents by entering the absorbance value of extract at 725 nm to the equation of the standard curve.

Total polyphenols content was also determined to pure poleo EO (2 μL + 100 μL anhydrous ethanol) and was used as control to calculate the release percentage of this oil. The test was performed in duplicate.

Mean recoveries of poleo oil were calculated by spiking empty microcapsules at 150 ppm by triplicate.

2.5. Microcosm assays. Incubation conditions

Shelled and natural peanuts intended for seed purposes (2013–2014 harvest) of two levels of seed germination (SG) (medium and low), with (F) and without fungicide treatment (WF) (10% thiophanate methyl + 1.33% Metalaxyl + Micronutrients -Co and Mo-) were used for this assay. Peanut kernels (225 g) were placed in plastic containers of 500 mL capacity. Poleo EO microcapsules were added at a dose of 5000 ppm and mixed to obtain a homogeneous distribution. This conteiners were incubated at room temperature (20–25 °C) for 114 d. The effect of microencapsulated poleo EO on peanut mycoflora and SG was evaluated at different times (0, 30, 80 and 114 d). The neutralization effect of the formulation was assayed by homogenizing microencapsulated poleo oil with natural peanuts during 10 min. All assays were performed in duplicate. Peanut seeds without capsules were used as control.

2.5.1. Estimation of mycoflora populations

A sample of 5 g of each treatment was taken, milled and shaken for 30 min with 45 mL of 1 g/L peptone: distilled water. Serial decimal dilutions until 10^{-3} were performed. An aliquot of 0.1 mL of each dilution was spread on the surface of two general counting media: DRBC (dicloran-rose bengal-chloramphenicol agar) and DG18 (dicloran 18% glycerol agar) (Pitt and Hocking, 1997; Samson et al., 2010). Plates were incubated in darkness at 25 °C for 5–7 d. Total fungal colonization was expressed as the mean of colony forming units developed in both media (DG18 and DRBC) per gram of peanut seeds (CFU/g). To determine the fungal count by genus, a number to each colony developed in both media (DG18 and DRBC) was assigned. Each numbered colony was subcultured in MEA plates. The colony identification numbers was clustered at genus level according to the macro and microscopic morphological characteristics (Samson et al., 2010; Samson and Frisvad, 2004). Finally, count of each fungal genus was stablished.

2.5.2. Effect on seed germination

Fifty seeds from each treatment were placed on plastic trays containing cotton and filter paper moistened with sterile distilled water. The trays were incubated at 25 °C and after of 5 days the number of seedlings in each treatment was determined. The results were expressed as percentage of seed germination (SG%).

2.6. Data analysis

Analysis of variance (ANOVA) was used to determine the impact of microencapsulated poleo oil, synthetic fungicide, SG initial level and storage period on total peanut mycoflora and SG. To establish significant differences, the test of Fisher's Least Significant Difference (LSD) ($p < 0.01$) was performed. Data were analyzed through the program InfoStat version 2012. InfoStat Group, FCA, National University of Cordoba, Argentina. http://www.infostat.com.ar URL.

3. Results

3.1. Encapsulation efficiency

Limonene (retention time (RT): 14.14 min) was the main component of pure poleo EO detected by GC/MS in this study. This component was detected at low levels (0.02%) in the surface of microcapsules. Therefore, high percentages of encapsulation of poleo EO were obtained (99.80%).

3.2. Release property of poleo EO microcapsules

The mean recovery percentage for poleo oil extraction was 102.0± 11.7%. A high content of total polyphenols (14.03 ± 0.12 mg GA/ 100 g vegetal material) was registered from pure EO. Fig. 1 shows the release of the poleo EO from the core of microcapsules under two storage conditions: alone and on peanut kernels. The greatest release of poleo EO (17%) was observed at 23 d regardless of temperature. Between 0.3 and 6.3% of poleo EO was detected outside of the capsules at the end of the evaluation period (78 d). In kernels conditioned at 0.95 a_W, 37% of poleo EO were outside of the microcapsules at the end of storage, while for lower a_W between 3 and 14% of oil were released at 78 d.

3.3. Effect on mycoflora

When the possible neutralization effect of microencapsulated poleo oil was estimated, total fungal counts were 2.6 and 3.1 Log_{10} CFU/g for control and treated peanut, respectively. Therefore, the antifungal effect of formulation was produced during the peanut incubation rather than the time of culture (5-7d), showing that microcapsules were diluted together with the peanut mycoflora.

Table 1 shows the level of total mycoflora (Log_{10} CFU/g) present in peanut seeds untreated and treated with poleo EO formulation, during a storage period of 114 d. According to ANOVA, fungal count was significantly affected ($p < 0.01$) by microencapsulated poleo oil (F: 97.62), time (F: 29.15) and its interaction (F: 11.23). Fungal reductions of 1.4 (F) and 2.0 (WF) Log_{10} CFU/g and 3.3 (F) and 5.4 (WF) Log_{10} CFU/g for low and medium SG respectively, were observed 30 d after formulation application. In the subsequent samplings (80 and 114 d), fungal counts continued to decline up to 2.5×10^1–1.2×10^2 CFU/g. Meanwhile, fungal counts in control seeds were 6.4×10^3 (F) and 1.8×10^4 (WF) CFU/g with low SG and 9.8×10^3 (F) and 2.1×10^6 (WF) CFU/g with medium SG.

Fungal genera most frequently isolated from control peanut seeds and that treated with poleo oil formulation are summarized in Table 2. *Penicillium* and *Aspergillus* were the main species isolated and fungal genera that showed a relatively low frequency of isolation were all included in the filamentous fungi group for ANOVA test. Sources of variation (microencapsulated poleo oil, synthetic fungicide, SG) and their interactions have been combined

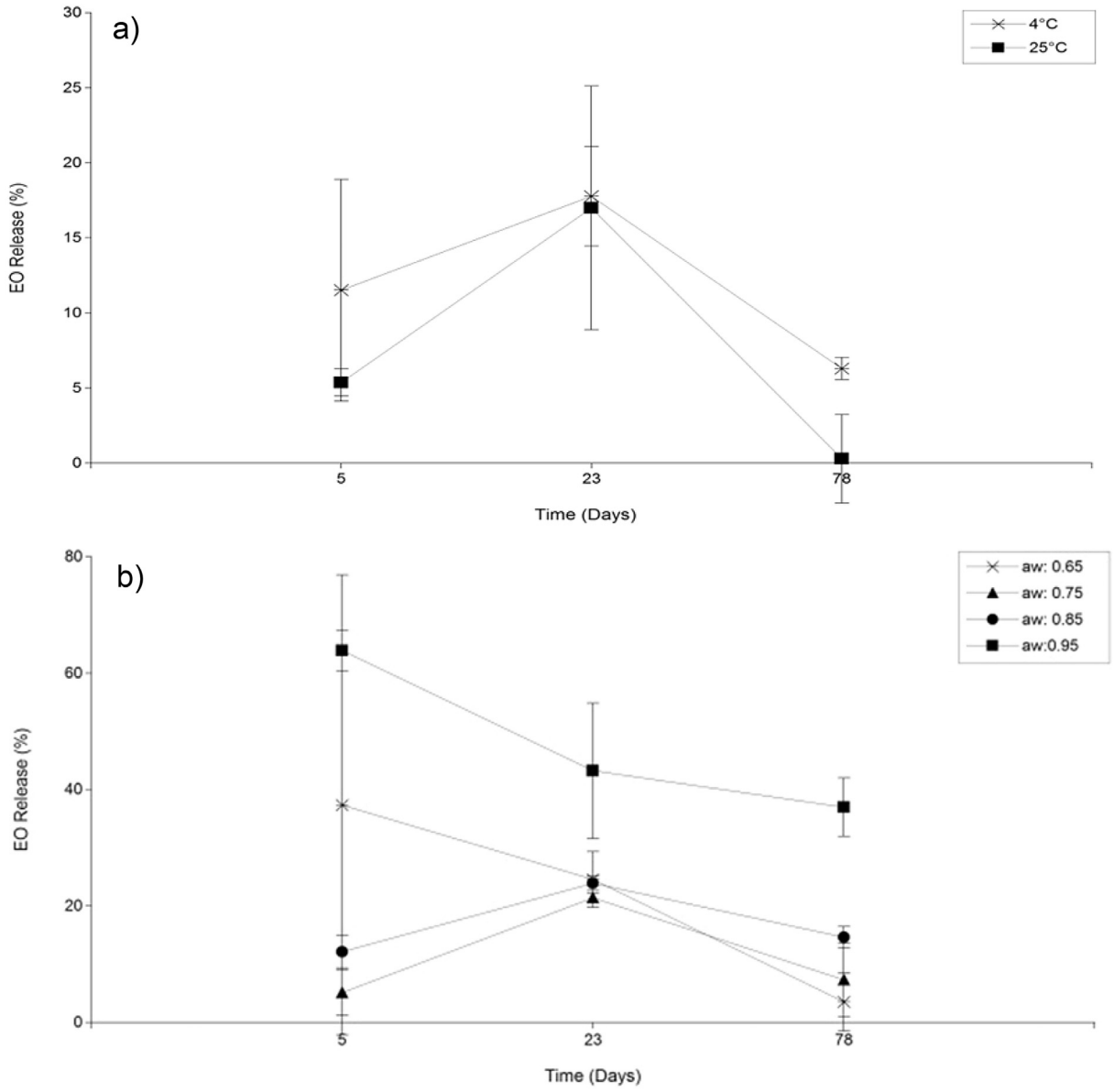

Fig. 1. Poleo essential oil (determined as total polyphenols) microcapsules release: a) stored at 25 and 4 °C; b) on peanut kernels conditioned at different a_W (activity water) levels.

Table 1
Effect of poleo essential oil formulation on peanut mycoflora.

SG	Fungicide	Treatment	Log_{10} CFU/g			
			t0	t1 (30 d)	t2 (80 d)	t3 (114 d)
Low	F	Control	3.25 ± 0.001b	3.32 ± 0.03b	2.10 ± 0.13a	3.77 ± 0.27b
		Poleo	3.25 ± 0.01a	1.85 ± 0.21a	1.40 ± 0.08a	0.85 ± 1.20a
	WF	Control	3.94 ± 0.12ab	4.15 ± 0.06b	2.90 ± 0.30a	3.76 ± 0.35ab
		Poleo	3.94 ± 0.12a	1.85 ± 0.21a	1.09 ± 0.55a	1.00 ± 1.41a
Medium	F	Control	4.18 ± 0.13a	3.56 ± 0.33a	4.56 ± 0.95a	3.12 ± 0.08a
		Poleo	4.18 ± 0.13a	0.85 ± 1.20a	1.30 ± 0.00a	1.20 ± 1.70a
	WF	Control	6.23 ± 0.03a	4.07 ± 0.19a	3.70 ± 0.88a	4.91 ± 2.16a
		Poleo	6.23 ± 0.03b	0.85 ± 1.20a	1.29 ± 0.16a	2.00 ± 0.42a

SG: seed germination, F: with fungicide treatment, WF: without fungicide treatment. Data with different letters for each treatment are significantly different according to LSD test ($p < 0.01$).

to determine the effect on predominant peanut fungal genera. There were no statistically significant effects of any of the factors studied on fungal genus isolation.

Penicillium species isolated from control and treated seeds throughout the storage period were classified into six sections: *Aspergilloides*, *Citrine*, *Divaricatum*, *Furcatum*, *Penicillium* and

Table 2
Fungal genera present in peanut seeds treated with poleo essential oil formulation and control.

Fungal genera (CFU/g)[a]	Control				Poleo			
	MSG		LSG		MSG		LSG	
	F	WF	F	WF	F	WF	F	WF
Ascomycetes								
Byssoclhamys	n.d.	n.d. - 5×10^2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eurotium	n.d.	n.d. - 2×10^3	n.d.	n.d. -1x10^3	n.d. - 1×10^2	n.d.	n.d.	n.d. - 1×10^3
Deuteromycetes								
Alternaria	n.d.	n.d.	n.d.	n.d.	n.d. - 3×10^2	n.d.	n.d.	n.d.
Aspergillus								
Can.d.idi	n.d.	n.d. - 1×10^3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Circun.d.ati	n.d. - 6×10^3	n.d. - 1×10^3	n.d. - 2×10^2	n.d.	n.d. - 6×10^3	n.d.	n.d. - 2×10^2	n.d.
Flavi	n.d. - 2×10^3	n.d. - 3×10^2	n.d. - 1×10^2	n.d. - 1×10^3	n.d. - 2×10^3	n.d. - 1×10^2	n.d. -1x10^2	n.d. - 1×10^3
Nidulantes	n.d. - 2×10^3	n.d.	n.d.	n.d.	n.d. - 2×10^3	n.d.	n.d.	n.d.
Restricti	n.d. -1x10^2	n.d. -1x10^4	n.d. - 1×10^2	n.d.	n.d.	n.d. - 1×10^4	n.d.	n.d.
Cladosporium	n.d. - 1×10^2	n.d.	n.d.	n.d. - 1×10^3	n.d. - 1×10^2	n.d.	n.d.	n.d. - 1×10^2
Fusarium	n.d.	n.d. - 1×10^2	n.d.	n.d. - 1×10^3	n.d. - 1×10^2	n.d.	n.d.	n.d. - 1×10^3
Metarizum	n.d. -1x10^3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Paecilomyces	n.d.	n.d. -1x10^3	n.d. -1x10^2	n.d.	n.d.	n.d.	n.d.	n.d.
Penicillium								
Aspergilloides	n.d. -1x10^4	n.d. - 1×10^2	n.d. - 1×10^2	n.d. - 2×10^3	n.d. - 1×10^4	n.d.	n.d. - 2×10^2	n.d.
Citrina	n.d. - 6×10^3	n.d. - 2×10^6	n.d. - 8×10^2	n.d. - 1×10^4	n.d. - 6×10^3	n.d. - 2×10^6	n.d. - 8×10^2	n.d. - 1×10^4
Divaricatum	n.d.	n.d.	n.d.	n.d. - 2×10^2	n.d.	n.d.	n.d.	n.d.
Furcatum	n.d. - 5×10^2	n.d.	n.d. -1x10^3	n.d. - 3×10^3	n.d. - 1×10^2	n.d.	n.d. -1x10^2	n.d. - 3×10^3
Penicillium	8×10^2 - 2×10^5	n.d. - 3×10^6	n.d. - 9×10^3	3×10^2- 1×10^4	n.d. - 5×10^3	n.d. - 2×10^2	n.d. - 3×10^3	n.d. - 8×10^3
Simplicia	n.d. - 3×10^3	n.d. - 4×10^4	n.d. - 2×10^2	n.d.	n.d. - 3×10^3	n.d.	n.d. - 2×10^2	n.d.
Ulocladium	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. - 2×10^2	n.d.	n.d.
Verticillum	n.d. - 4×10^3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. - 1×10^2	n.d.
Levadura	n.d.	n.d.	n.d. - 2×10^2	n.d.	n.d.	n.d.	n.d.	n.d.
Zygomycete								
Absidia	n.d.	n.d.	n.d.	n.d.	n.d.	n.d. - 1×10^2	n.d.	n.d.
Unidentified	n.d. - 1×10^2	n.d. - 1×10^4	n.d. - 4×10^3	n.d. - 1×10^4	n.d. - 2×10^2	n.d. - 1×10^4	n.d. - 7×10^2	n.d. - 2×10^2

[a] Range of three replicates in DRBC and DG18 media. n.d.: <1×10^2 CFU/g. Genera and Sections according to Samson and Frisvad (2004) and Samson et al. (2010). MSG: medium seed germination. LSG: low seed germination. F: with fungicide treatment, WF: without fungicide treatment.

Simplicia. Poleo EO formulation reduced between 0.0 and 25.6% the growth of *Penicillium* species in peanut seeds with medium SG, and between 4.5 and 11.7% in seeds with low SG. The most species of *Aspergillus* genus were isolated from seeds with medium SG, including species of *Circumdati*, *Flavi*, *Nidulantes*, *Restriciti* and *Candidi* sections. Microencapsulated oil was able to reduce between 0.0 and 13.2% the growth of *Aspergillus* species. Meanwhile, the development of filamentous fungi group decreased in the order of 4.46–20.13% by the presence of poleo oil formulation in peanut seeds.

3.4. Effect on seed germination

Table 3 shows the percentage of SG of peanut seeds treated with the poleo EO formulation compared to the control, during a storage period of 114 d. According to the ANOVA test, SG was significantly affected ($p < 0.01$) by microcapsulated poleo oil (T) (F: 139.66), synthetic fungicide (F) (F: 135.05), initial level of SG (SG) (F: 64.76) and by F*T (F: 57.72), SG*T (F: 31.99) and time*T (F: 15.57) interactions.

Poleo EO formulation significantly reduced ($p < 0.01$) the percentage of seedlings (between 84 and 100%) at the first sampling period (30 d), regardless of the SG level previously established (low or medium) and the presence of synthetic fungicide (F and WF). At the end of the storage period (114 d), the 100% of the seeds treated with the formulation were unable to germinate. While the SG of the control treatments ranged between 12 and 60% for seeds with fungicide and between 0 and 20% for seeds without fungicide.

4. Discussion

The gelatin/gum arabic system followed by lyophilization process was effective to encapsulate poleo EO with high EE% (99.8%). Similar results (EE ranged from 96.1% ± 1.7 to 98.3± 0.3%) were

Table 3
Effect of poleo essential oil formulation on the percentage of germinated seeds.

Initial SG	Fungicide	Treatment	SG (%)			
			t0	t1 (30 d)	t2 (80 d)	t3 (114 d)
Low	F	Control	24.67 ± 2.83a	22.00 ± 5.66a	23.00 ± 9.90a	23.00 ± 15.60a
		Poleo	24.67 ± 2.83b	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
	WF	Control	5.34 ± 1.89a	4.00 ± 2.83a	0.00 ± 0.00a	1.00 ± 1.41a
		Poleo	5.34 ± 1.89b	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
Medium	F	Control	43.34 ± 4.72a	56.00 ± 19.80a	55.00 ± 1.41a	51.00 ± 12.7a
		Poleo	43.34 ± 4.72b	0.00 ± 0.00a	0.00 ± 0.00a	0.00 ± 0.00a
	WF	Control	12.00 ± 1.88a	11.00 ± 7.07a	20.00 ± 5.66a	17.00 ± 4.24a
		Poleo	12.00 ± 1.88b	2.00 ± 2.83a	1.00 ± 1.41a	0.00 ± 0.00a

SG: seed germination, F: with fungicide treatment, WF: without fungicide treatment. Data with different letters for each treatment are significantly different according to LSD test ($p < 0.01$).

obtained when a mixture of oleoresin paprika and soybean oil was microencapsulated by complex coacervation using glutaraldehyde or transglutaminase as crosslinking agents; lyophilized or sprays dried (Alvim and Grosso, 2010).

When the release of the poleo oil from the microcapsule cores was evaluated, it was notable that low, but constant levels (0.3–17.7%) were recorded during the storage period (78 d), regardless of temperature conditions assayed. Although the release test of poleo oil by the technique of polyphenols was evaluated in sealed systems, not a cumulative increase of these molecules was observed in the surrounding atmosphere. Haroun and El Toumy (2010) demonstrated that the involvement of hydrogen bonding and hydrophobic interactions are the major forces involved in the stabilization of gelatin-based polymeric biocomposite film by the plant polyphenols (catechin and gallic acids derivatives). These authors also studied the thermal stability of crosslinked gelatin-based composite film and observed that the *Acacia nilotica* extract stabilized the gelatin molecules leading to moderate increase of the denaturation temperatures relative to the uncrosslinked one. Therefore, it seems appropriate to store the poleo oil formulation at 4 °C. When microcapsules were added to peanut kernels, on the one hand it was observed the similar behavior to the systems that contained pure microcapsules recording residual poleo levels in the order of 3.5–63.8% throughout the storage time. In this case, not only the protein "gelatin" was present in the system, but also those contained in the peanut kernel. Polyphenols act as potent inhibitors of ROS-generating enzymes such as xanthine oxydase (Cos et al., 1998), cyclooxygenase and lipoxygenase (Kim et al., 1998), by complexing the proteins. On the other hand, poleo oil levels in the peanut surrounding atmosphere were between 18 and 58% higher at 0.95 a_W than at lower a_W. This behavior could be due to a destabilization of capsule walls by the gelatin humectation giving a minor stiffness to the microcapsules and therefore easing the oil release. Thus, the increase of grain a_W promoted the release of encapsulated poleo EO. Similarly, Passone and Etcheverry (2014) observed that the residue of poleo oil applied as volatile phase on peanut system were 33.3% higher at the lowest a_W tested (0.93) than at 0.98 a_W, regardless of incubation time. Nevertheless in that previous work, oil residues were evaluated on grains contaminated with *Aspergillus* section *Flavi*, so under favorable conditions for fungal metabolism the oil may have being degraded. Moreover, it is important to highlight that poleo oil residual levels were reduced in the order of 26.6–99.7% at 35 d of incubation, regardless of the conditions assayed (Passone and Etcheverry, 2014).

The poleo oil formulation showed a significant antifungal effect on peanut mycoflora, with reductions between 59.2 and 77.4% at 5000 ppm. These reductions were greater than those reported by Passone and Etcheverry (2014) who evaluated the antifungal effect of pure poleo EO on *Aspergillus* section *Flavi* in peanut conditioned at 0.95 and 0.98 a_W (46.8 and 44.9%, respectively). It is also important to emphasize that, if fungal count results are compared between the treatment with microencapsulated poleo applied to WF seeds and the F control is clearly observed that antifungal effects produced by the formulation were in the order of 57.1 and 63.2% higher for seeds with low and medium SG, respectively. Mycological studies showed a prevalence of *Penicillium* and *Aspergillus* lesser extent in peanut seeds throughout the storage period. Others studies in Argentina found similar results in peanut mycoflora composition (Magnoli et al., 2006; Passone et al., 2014). This may be because water availability of grains in natural experimental conditions ranging between 0.64 and 0.79 a_W throughout the storage period, so only xerophilic populations as *Penicillium* and *Aspergillus* can be maintained (Magan and Aldred, 2007).

The research of this study also showed that poleo EO microcapsules have an inhibitory effect on seed germination of peanut.

According to the data at present available the inhibition of peanut germination due to use of EOs had not been reported. De Lira Guerra et al. (2015) observed no inhibition of germination in peanut seeds treated with seven EOs (*Lemongrass martinii* (Roxb.) Stapf var. motia Burk, *Cedrus atlantica* Manetti, *Copaifera officinalis* L., *Zingiber officinale* L., *Eucalyptus staigeriana* F. (Muell), *Juniperus communis* L., and *Ocimum basilicum* L.) at 1000 ppm. However, the oil/floral water mixture of *Justicia anselliana* produced peanut seed germination reductions of 9.4% (Salomé Kpoviessi et al., 2009). Palacios et al. (2010) showed that ethanol extracts of *L. turbinata* slightly inhibited the germination of seeds of *Avena sativa* (13.79%) and *Raphanus sativus* (29.21%). The detrimental effect of EOs on SG can be attributed to the high content of monoterpene compounds. Previous researches showed that monoterpene compounds and EOs possess potent herbicidal effects on weed germination and seedling growth of various plant species (Kordali et al., 2007; Singh et al., 2002, 2004; Tworkoski, 2002; Zunino and Zygadlo, 2004). According to Pascual et al. (2001) and Passone and Etcheverry (2014), the monoterpene limonene was the main component of poleo oil detected in this work. Limonene extracted from leaves of *Citrus aurantium* L. inhibited the growth of *Amarantus retroflexus* (Al-Saadawi et al., 1985). However, limonene in the concentration range 0.1–10 mM was inactive on seed germination and root growth of primary maize seeds (Abrahim et al., 2000).

Peanut kernels intended for seed are generally stored in shell until planting time, so it would be interesting to assess whether changing the application mode could prevent unwanted effect on seed germination.

5. Conclusion

Therefore this work clearly demonstrated for the first time that (i) poleo EO was encapsulated through the gelatin/ gum Arabic system with high efficient percentages; (ii) detectable levels of poleo oil were recorded in the surrounding atmosphere of peanut kernels even at 78 d of storage; (iii) the microencapsulation process did not affected the antifungal power of this natural compound facilitating its application in the peanut storage system; (iv) adverse effects of the poleo formulation were observed on peanut seeds germination.

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