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Microencapsulation of *Peumus boldus* oil by complex coacervation to provide peanut seeds protection against fungal pathogens

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ABSTRACT

In this study, *Peumus boldus* Mol. (boldo) essential oil (EO) microcapsules were prepared using the complex coacervation technique with gelatin/gum arabic, in order to enhance the dwell period of this preservative in the storage system. Bloom gelatin 240 – formaldehyde 10 min were the optimal encapsulation conditions (94.38%). The microcapsules prepared under optimal conditions had an average diameter of 5.67 μm. Boldo EO release was very low at 12 days of incubation (1×10^{-4}%) and was not affected by temperature. In grains, 99.9% of EO remained encapsulated at the end of the incubation period (10 days), including at the highest a_W tested (0.95). Boldo EO formulation reduced between 31.5 and 42.5% the peanut mycoflora being *Penicillium* and *Aspergillus* the main species isolated from control and treated seeds. However, volatile compounds released by the formulation decreased the germination capacity of peanut seeds. To avoid this effect, the implementation of boldo EO formulation on stored shell peanut will be evaluated in future studies.

In conclusion, boldo EO was encapsulated with high percentages of efficiency by complex coacervation keeping the antifungal ability. The application of boldo EO formulation could increase seed protection against fungal pathogens during a storage period of 114 days, if it possible to reduce the allelophatic effect.

1. Introduction

Peanut (*Arachis hypogaea* L.) is an important commodity for the economy in Argentina. Production in the 2014/2015 crop season was 1 million tons (SIIA, 2015). However, considerable yield losses in peanut are caused by diseases. Some parasitic fungi feed on plants such as peanuts, penetrating actively or through openings in the host (Zorzete et al., 2011), which may attack the seeds at different maturation stages (Atayde et al., 2012). Peanut seeds infected by seed-borne fungi have been reported to produce seed abortion, shrunken or size reduced seeds, seed rot, necrosis or discoloration and reduction of germination capacity by physiological alternations (Lomate and Hivrale, 2011). Because of the particular growth that have the peanut seeds (hypogeo) and as consequence of consecutive peanut monoculture, the prevalence of fungal pathogen infections continues to increase (Li et al., 2014).

Therefore, pre harvest peanut seeds contain mycelia and spores of pathogenic fungi, which can result in a significant decrease in grain quality during the storage (Passone et al., 2014b). Optimum environmental conditions (temperature and humidity controlled) in peanut storage system is important to prevent the typical storage fungi colonization (*Penicillium* spp., *Aspergillus* spp., *Eurotium* spp), which may affect the seed germination and subsequent field emergency (Passone et al., 2014a). Fungicides are important tools for managing diseases in agricultural crops, being treatments at seed level, the convenient manner to reach the crop protection. These fungicides are applied at pre-seedling time; while during the peanut storage period of about 4–5 months (from harvest to planting), seeds do not receive any treatment. Although, seed treatments can be particularly useful, since they can provide protection to young plants during a vulnerable stage in their development (Walters et al., 2013), the fungicides used are hazardous to the human health and environment (Silva and Fey, 2006). Therefore there is considerable interest in finding alternatives to chemical pesticides for suppression of soil-borne plant pathogens.

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Peumus boldus Mol. (boldo) is a native tree from the central region of Chile, being part of the sclerophyllous forests, characteristic of the Mediterranean climate (Vogel et al., 2011). The phenolic constituents (mainly proanthocyanidins and flavonol glycosides), alkaloids and essential oils (EOs) are the main bioactive compounds of boldo leave extracts (Passone et al., 2014a; Simirgiotis and Schmeda-Hirschmann, 2010). The EOs are aromatic oily liquids obtained by hydrodistillation from plant materials (whole tissues or seed) and are usually mixtures of several components. The fact that they are constituted by a great variety of compounds confers on them other advantages, such as different modes of action depending on the involved compound, the ability to attack different genera and that hinders the development of resistance by the pathogen (da Cruz Cabral et al., 2013). Also, they have attracted increasing interest in recent years, because to the GRAS status conferred by the Food and Drugs Administration (FDA) and its easy decomposition that become them in environmentally friendly (da Cruz Cabral et al., 2013). Boldo EO (volatile components) has proven effective in controlling aflatoxicogenic mycoflora and aflatoxin accumulation in culture medium (Passone et al., 2013) and on irradiated and natural peanut grains *in vitro* (Passone et al., 2014a). The fumigant property of EOs is very interesting and valuable, especially because it can have great practical implications for the pest treatment in that places of the difficult access where they normally cannot be reached by conventional sprayings. However, the volatilization rate of the EO is generally affected by environmental conditions (Passone et al., 2014a). Whereby, this process can be very poor and therefore ineffective or excessive and produce uncomfortable effects. Microencapsulation is an effective technology for protection of the stability and activities of polyphenols (Wang et al., 2016). The sustained-release agents significantly improve efficacy, reduce the administration frequency, increase safety, and reduce environmental pollution (Wu et al., 2015). Encapsulation is a process in which thin film, generally of polymeric materials is applied to little solid particles, liquid or gases droplets (Kaushik et al., 2016). The process of complex coacervation is recognized as one of the most promising technologies for stabilization of omega-3 oils by microencapsulation, while delivering a high payload (40–60%) (Barrow et al., 2007).

Therefore, the aims of this study are (i) to prepare and optimize boldo EO microcapsules by using complex coacervation method, and (ii) to research the effect of boldo microcapsule formulation on peanut seeds.

2. Materials and methods

2.1. Collection and characterization of the essential oil

The spice used in this study (*Peumus boldus* Mol (boldo)) was collected in December-March period 2014 in the O'Higgins Region (VI), Chile. Dried leaves (0.3373 ± 0.004 a_W) of boldo were purchased from a local market located in La Paz town, Córdoba, Argentina. A portion (100 g) of each plant material part 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 2.0%). The obtained EO was dried over anhydrous sodium sulfate and, after filtration, stored in a sterilized vial at 4 °C for up to 1 week until testing. The characterization of boldo EO was performed by gas chromatography mass spectrometer (GC/MS) (Passone and Etcheverry, 2014).

2.2. Preparation of coacervate microcapsules

Microcapsules were made by complex coacervation following Girardi et al. (2015) and Liu et al. (2010) with some modifications.

Table 1
D-optimal design and their experimental results.

Run order	X_1	X_2	X_3	Response
1	60.00	GLUT	240	81.02
2	120.00	FORM	220	97.54
3	37.00	FORM	220	87.64
4	120.00	GLUT	240	67.01
5	92.00	GLUT	220	83.46
6	120.00	FORM	220	96.97
7	10.00	GLUT	220	93.87
8	10.00	FORM	240	94.12
9	120.00	GLUT	240	77.88
10	10.00	FORM	240	96.58
11	10.00	GLUT	240	85.54
12	37.00	FORM	240	88.56
13	10.00	FORM	220	95.83
14	10.00	GLUT	240	84.01
15	10.00	FORM	220	94.12
16	120.00	FORM	240	93.05
17	120.00	GLUT	220	82.94
18	92.00	FORM	220	85.86
19	37.00	GLUT	220	76.96

Boldo EO was used as the core material, while gelatin and gum arabic were used as the wall material. Twenty five mL of gelatin and gum arabic aqueous solution 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 1 M (NaOH). 450 μL of boldo EO were 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 1 M (HCl) and the stirring was continued for 10 min.

Subsequently, pH was adjusted to 9 with NaOH 1 M and stirring others 10 min. After that temperature was lowered to 10 °C in an ice bath and 5 mL of crosslinking agent was added to compact the gelatin/gum arabic coating. Finally, microcapsules were washed twice with distilled water and were stored at −20 °C until the lyophilization step.

For lyophilization process, microcapsules were frozen at −80 °C during 3 h and then were lyophilized (L-T8-A-B3-CT, RIFICOR). Then the samples were ground (CT 193 Cyclotec™ Sample Mill) to obtain a powder with a particle size of 1000 μm and stored at −20 °C.

2.3. Experimental design

Experimental design response surface methodology (RSM) coupled with D-optimal design was employed to optimize and investigate the effects of microencapsulation conditions on the efficiency of encapsulation. The D-optimal method gives lower numbers of experiments respect to other design methods and can handle categorical factors included in the design of experiment (Kuram et al., 2013). The experimental design used in this study considered reticulation time (X_1) as a numerical factor, and the crosslinking agent (X_2) and gelatin bloom (X_3) as categorical factors. Table 1 shows the run order of experimental design and the observed response (Efficiency of Encapsulation (EE)) for the three variables; five levels to the numerical factor were applied, with 19 experimental runs generated.

A quadratic polynomial, including all interaction terms, was used to calculate the response:

$$Y = \beta_0 + \beta_0 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{12} X_1{}^2 X_2 + \beta_{12} X_1 X_2{}^2 + \beta_{13} X_1{}^2 X_3 + \beta_{13} X_1 X_3{}^2 + \beta_{23} X_2{}^2 X_3 + \beta_{23} X_2 X_3{}^2 + \beta_{11} X_1{}^2 + \beta_{22} X_2{}^2 + \beta_{33} X_3{}^2 + \varepsilon \quad (1)$$

where Y is the predicted response variable (EE), β_0 is the intercept term, $\beta_{1\text{-}3}$ are the linear coefficients, β_{12}, β_{13} and β_{23} are the inter-

action coefficients, β_{11}, β_{22} and β_{33} are the quadratic coefficients, and X_1 to X_3 are the coded independent variables.

2.4. Efficiency of encapsulation

Efficiency estimation was adapted from Kaushik and Roos (2007). 0.5 g of microcapsules was added to 10 mL of chloroform in glass flask and shaken for 5 min. Powder particles were separated from chloroformic extract by filtration through filter paper (WhatmanN° 1). Absorbance of chloroformic extracts was measured with a spectrophotometer (Spectrum SP 2100UV) at 250 nm by using pure chloroform as blank. This wavelength was found to correspond to the maximum absorbance of compounds over the spectrum of wavelengths from 200 to 600 nm. A standard curve was building by measuring absorbance of boldo EO dissolved in chloroform at different concentrations (v/v). The encapsulated boldo EO was determined as the difference between the initial level and the estimated value on the microcapsule surface. EE was expressed as the percentage of boldo oil encapsulated respect to the initial amount of EO. The test was performed in triplicate.

EE% of the microcapsules performed on the optimal conditions was confirmed by gas chromatography coupled to a 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) according to Passone and Etcheverry (2014). 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 5.0–49.6 psi. The temperature of the injector and GC transfer line was maintained at 250–200 °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 EO was performed by comparison with spectra libraries NIST MS Search 2.0.

2.5. Optical microscopy

Size and morphology of the microcapsules were examined by optical microscopy at 40 X magnification (Carl Zeiss, 37081) and photographed using Motic Images Plus 2.0 (2005) software.

2.6. Release properties of microencapsulated boldo EO

Release of boldo EO from microcapsules performed with gelatin bloom 240 and formaldehyde 10 min as crosslinking agent (240 − F10) was evaluated *in vitro*. To characterize the formulation stability, 0.1 g of formulation was placed in flaks, which were then sealed and incubated at 25 and 4 °C for 12 days. To evaluate the release of microencapsulated boldo oil on the substrate, 0.1 g of microcapsules was placed into a flask containing 5 g of sterile peanuts conditioned at 0.65, 0.85 and 0.95 a_W which were sealed and incubated at 25 ± 2 °C for 10 days. In both assays the boldo EO residua were determinate at different times. For that, treatments were exposed to the solid phase microextraction fiber (SPME) for 30 min at room temperature. Fiber was remained in the injector port for 10 min under the same conditions described in Section 2.3 for GC–MS analysis.

For quantification of the residual oil, an external calibration curve of eucalyptol (MW: 154.25 g/mol; CAS: 470-82-6) was used. The quantification curve in the range of sample concentrations (0.00303085–1.21155 μg μL^{-1} of eucalyptol; $R^2 = 0.984$) 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 eucalyptol.0.05 and 0.30 ng/g.

2.7. Microcosm assays. Incubation conditions

Shelled and natural peanut intended for seed purposes (2013–2014 harvest) of two levels of germination (GP) (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. 225 g of peanut kernels were placed in plastic flasks of 500 mL capacity. Boldo EO microcapsules (240 − F10) were added at a dose of 3000 ppm and mixed to obtain a homogeneous distribution. Plastic flasks were incubated at room temperature (20–25 °C) for 114 days. The effect of microencapsulated boldo EO on peanut mycoflora and germination power (GP) was evaluated at different times (0, 30, 80 and 114 days). The assay was performed in duplicate. Peanut seeds without capsules were used as control.

2.7.1. Estimation of mycoflora populations

A sample of 5 g was taken from each treatment, 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 surface of two general counting media: DRBC (dicloran- rose bengal- chloramphenicol agar) and DG18 (dicloran 18% glycerol agar) (Pitt and Hocking, 1997). Plates were incubated in darkness at 25 °C for 5-7 days. Colonization of peanut seeds was expressed as colony forming units per gram of peanut seeds (CFU/g). The identification of fungal genera was done according to Samson and Frisvad (2004), Samson et al. (2010). *Aspergillus* and *Penicillium* sections were identified following the identification keys of Pitt (2000), Houbraken et al. (2011, 2014), Visagie et al. (2014a,b) and Samson and Frisvad (2004), Samson et al. (2010).

2.7.2. Effect on germination power

50 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 for 5 days. The percentage of germinated seeds in each treatment was determined and the results expressed as% GP.

2.8. Data analysis

The experimental data obtained were subjected to multiple regression analysis using the Design Expert software package version 9 to obtain the coefficients of the quadratic equation. The multiple correlation coefficient (R) and coefficient of determination (R^2) were calculated to evaluate the performance of the regression equation. The performance of the model in the experimental area was investigated graphically. Statistical evaluation of the RSM model was carried out using analysis of variance (ANOVA), which is required to test the significance of the developed model equation.

Analysis of variance (ANOVA) was used to determine the impact of different encapsulation conditions on the size of the microcapsules and the encapsulation efficiency. Significant differences were establish by performed Di Rienzo, Guzman and Casanoves test (DGC) ($p < 0.01$). ANOVA was also used to determine the impact of fungicide seed treatment, initial level of GP, storage period and microcapsule treatment on total peanut mycoflora and GP. 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,

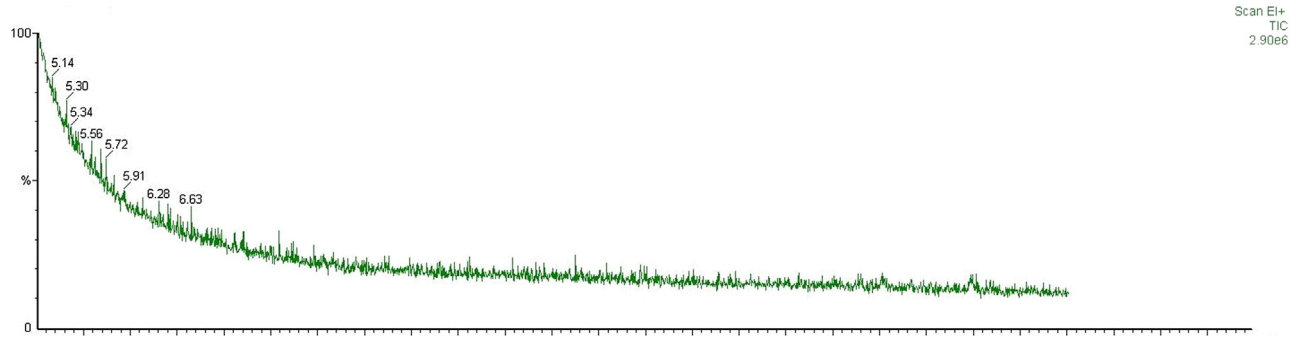

Fig. 1. GC/MS chromatogram corresponding to chloroformic extracts of boldo EO microcapsules 240 – F10.

Table 2
ANOVA regression and lack of fit.

Source	Sum of squares	df	MS	*F* value[a]	*p*-value
Model	467.96	6	77.99	79.26	<0.0001**
A	55.50	1	55.50	56.40	0.0001**
B	287.12	1	287.12	291.79	<0.0001**
AB	57.03	1	57.03	57.96	0.0001**
BC	26.69	1	26.69	27.12	0.0012*
A2	105.34	1	105.34	107.06	<0.0001**
A2C	54.44	1	54.44	55.33	0.0001**
Residual	6.89	7	0.98		
Lack of Fit	1.08	3	0.36	0.25	0.8601

df: degrees of freedom; MS: means of squares.
[a] Snedecorís F test.
*Significant t $p \leq 0.05$.
*Highly significant at $p < 0.001$.

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3. Results

3.1. Optimization of microencapsulation conditions

Statistical results of the RSM model indicated that cubic model was found to be aliased. Linear and interactive (2FI) models exhibited high *p*-values and lower *F*-values. Therefore the quadratic model (low *p*-value and higher *F*-value) was chosen to describe the effects on EE (Table 2).

The obtained results indicate a good and adequate agreement between D-optimal experimental data and actual data.

It is very important check that the analysis of adequacy of developed mathematical model there has been no poor results. So that, by constructing analytical plots the adequacy of developed mathematical model was investigated: 1) Normal probability plot of the studentized residuals to check for normality of residuals. 2) Studentized residuals versus predicted values to check for constant error. 3) Externally studentized residuals to look for outliers, i.e., influential values. 4) Box-Cox plot for power transformations.

Finally, by applying coefficients to the generalized second order polynomial model to predict the EE, the following model was obtained:

$$Y = 80.37 - 2.22 * A - 5.16 * B - 2.25 * A * B - 1.42 * B * C + 9.68 * A2 - 2.23 * A2 * C \quad (2)$$

Where, Y is response to optimize (EE), A represents the numerical factor (crosslinking time), B and C categorical factors (Crosslinking agent and gelatin bloom), respectively.

Thus, second order polynomial equation was developed with high correlation coefficient value (R^2: 0.9855). The optimal conditions were found to be crosslinking agent: formaldehyde, crosslinking time: 10 min and gelatin bloom: 240. Under this optimal scheme, EE was 94.38%.

When the EE of optimal conditions was confirmed by GC/MS any components of boldo EO were detected in the chloroformic extracts (Fig. 1), therefore the encapsulation was totally efficient.

3.2. Size and morphology of the microcapsules

The light microscopy photographs of the boldo EO microcapsules by coacervation prepared under optimal conditions are shown in Fig. 2. Microcapsule size analyses before (6.97 ± 3.77 μm) and after (4.33 ± 2.19 μm) freeze dried clearly demonstrates that it was affected by liophylization process, but this reduction was not statistically significant (*p*: 0.0026 and F: 9.98).

3.3. Release properties of microencapsulated boldo EO

Results on the stability of the formulation showed a very low release (1×10^{-4}%) of boldo EO at 12 days of incubation and this behavior was not affected by the temperature (Fig. 3.a).

Low EO levels were also observed in the peanut surrounding atmosphere at the end of the storage period (10 days) (Fig. 3.b).

Table 3
Effect of boldo EO formulation on mycoflora (Log_{10} CFU/g).

GP	Fungicide	Traetment	Log_{10} CFU/g			
			t0	t1 (30 d)	t2 (80 d)	t3 (114 d)
Low	F	Control	3.25 ± 0.01b	3.32 ± 0.03b	2.10 ± 0.13a	3.77 ± 0.27b
		Boldo	3.25 ± 0.01b	0.00 ± 0.00a	2.54 ± 0.91b	2.55 ± 0.21b
	WF	Control	3.94 ± 0.12ab	4.15 ± 0.06b	2.90 ± 0.30a	3.76 ± 0.35ab
		Boldo	3.94 ± 0.12a	0.85 ± 1.20a	2.51 ± 0.72a	2.57 ± 0.04a
Medium	F	Control	4.18 ± 0.13a	3.56 ± 0.33a	4.56 ± 0.95a	3.12 ± 0.08a
		Boldo	4.18 ± 0.13a	1.00 ± 1.41a	3.55 ± 1.48a	1.50 ± 0.28a
	WF	Control	6.23 ± 0.03a	4.07 ± 0.19a	3.70 ± 0.88a	4.91 ± 2.16a
		Boldo	6.23 ± 0.03b	2.48 ± 0.00a	2.60 ± 0.19a	2.82 ± 0.4a

Data with different letters for each treatment are significantly different according to LSD test. ($p > 0.01$).

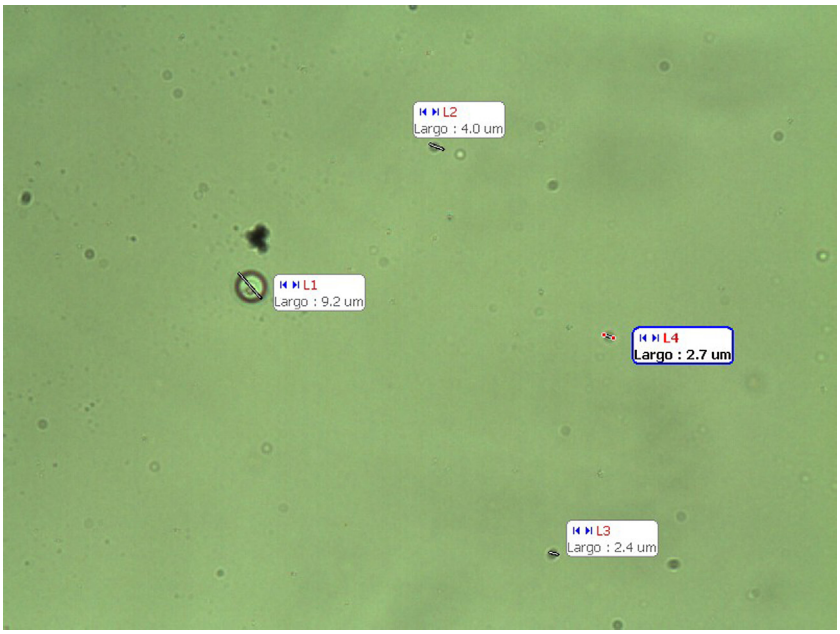

Fig. 2. Photograph of boldo EO microcapsules freeze-dried 240 – F10 (40 X).

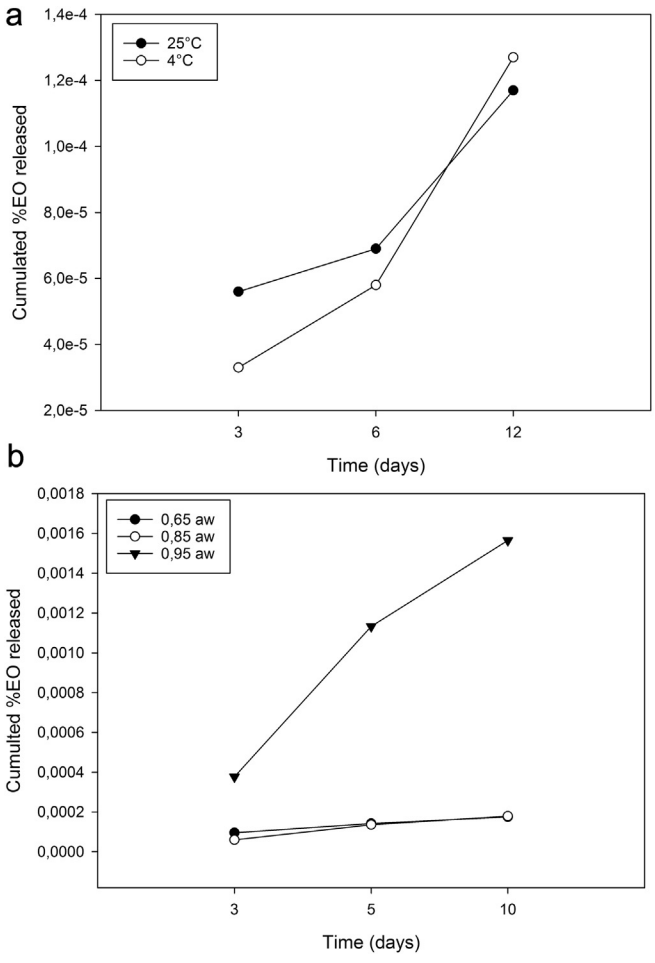

Fig. 3. Release of boldo EO from microcapsules: a) stored at 25 and 4 °C; b) on peanut kernels conditioned at different a_W levels.

The release rate of microencapsulated EO was higher at 0.95 a_W than at the two lowest a_W (0.65 and 0.85). However 99.9% of boldo EO remained inside de capsules.

3.4. Effect on mycoflora

Table 3 shows the mycoflora incidence (Log_{10} CFU/g) of peanut seeds treated and untreated with boldo EO formulation through a storage period of 114 days. ANOVA revealed that fungal counts were significantly affected ($p > 0.01$) by the treatment (F: 50.61), GP (F: 24.91), time (F: 24.66), and treatment*time interaction (T * t) (F: 12.78). Boldo EO formulation reduced total fungal counts at 30 days of incubation, both in seeds with (F) (3.0–3.2 $\log_{10}$ CFU/g) and without fungicide (WF) (3.0–3.7 $\log_{10}$ CFU/g). In the subsequent sampling periods (80 and 114 d) fungal counts for seed with boldo EO formulation were between 3.5×10^1 and 8.1×10^2 CFU/g. While fungal counts estimated for control seeds ranged between 1.3×10^3 and 1.3×10^6 CFU/g at 114 days of storage. The boldo oil formulation (microencapsulated boldo applied to WF seeds) was between 29.5 and 50.7% more effective than fungicide (F applied to control seeds) in controlling peanut mycoflora.

Fungal genera most frequently isolated from control peanut seeds and treated with boldo oil formulation are summarized in Table 4. *Penicillium* and *Aspergillus* were the main species isolated, while fungal genera that showed a relatively low frequency of isolation were all included in the filamentous fungi group for ANOVA test. Sources of variation for predominant peanut fungal genera have been evaluated germination power (PG), microcapsule treatment, fungicide seed treatment and their interactions. 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 *Simplicia*. Boldo EO formulation reduced between 56 and 100% species of *Simplicia* section (*P. pinophillum, P. miniolution, P. funiculosum*) in peanut seeds with medium PG, while in seeds with low PG only. *P. funiculosum* was able to develope (Fig. 4a). *P. crustosum* represents 40.9% of the isolates of *Penicillium* section. This fungal specie was controlled between 51 and 89% with boldo oil formulation. *P. vari-*

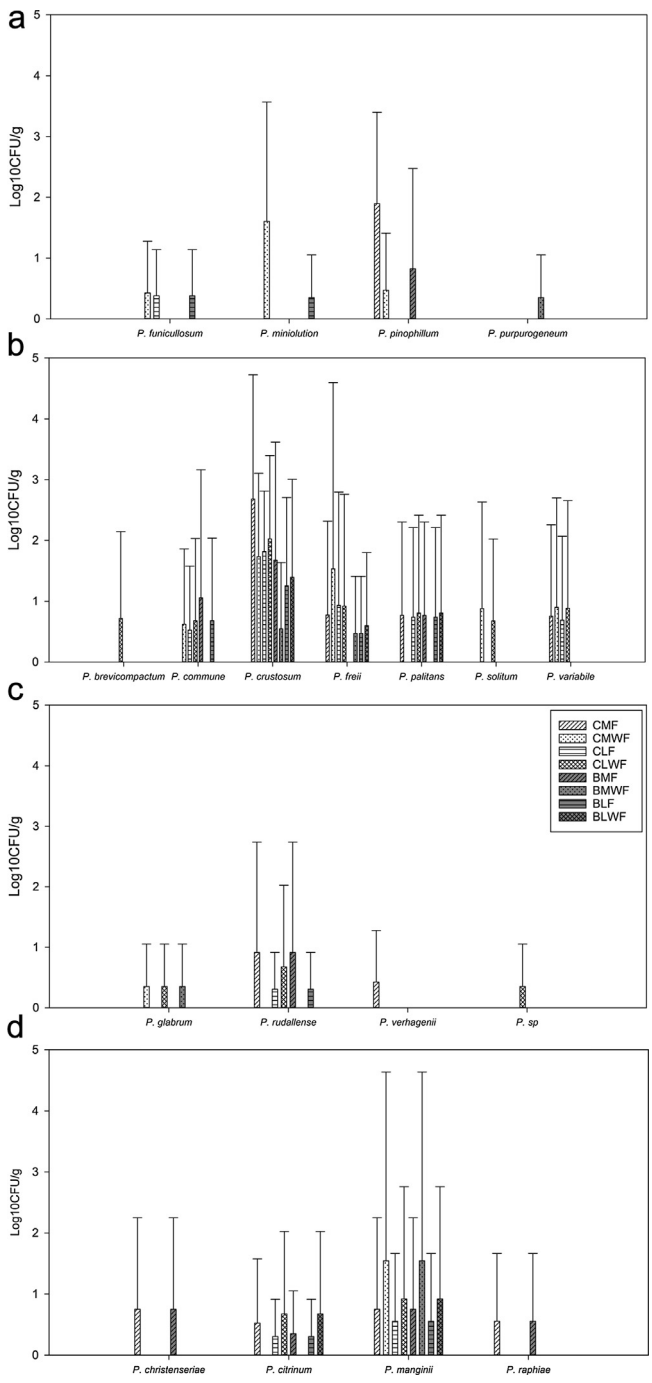

Fig. 4. Effect of boldo EO formulation on *Penicillium* section (a) *Simplicia,* (b) *Penicillium*, (c) *Aspergilloides and* (d) *Citrina.* CMF: Control: Medium GP: F; CMWF Control: Medium GP: WF; CLC: Control: Lower GP: F; CLWF: Control: Lower GP: WF; PMF: Poleo: Medium GP: F; PMWF: Poleo: Medium GP: WF; PLF: Poleo: Lower GP: F; PLWF: Poleo: Lower GP: WF.

abile, P. brevicompactum and *P. solitum* were completely inhibited, while the isolation of *P. freii* and *P. commune* declined between 61 and 100% in treated seeds. *P. palitans* was the only specie of this section on which the formulation had no effect (Fig. 4b). *P. rudallense*, *P. verhagenii* and *P. glabrum* were the species from *Aspergilloides* section. The effect of boldo EO formulation on these isolates was selective, since it generated inhibition in some cases while in others no (Fig. 4c). Species belonging to *Citrine* section (*P. christenseriae*, *P. manginii*, *P. citrinum* and *P. raphiae*) were resistant to the boldo oil microcapsules (Fig. 4d).

The most *Aspergillus* species isolated belonged to *Circumdati* (*A. ochraceus*), *Flavi* (*A. caelatus*, *A. flavus*, *A. leporis*, *A. nomius*, *A. parasiticus*) *Nidulantes* (*A. versicolor*), *Restriciti* (*A. penicilloides*) and *Candidi* (*A. candidus*) sections (Fig. 5). *A. penicilloides* and some species from *Flavi* section (*A. parasiticus* and *A. leporis*) were totally inhibited by boldo EO formulation. Moreover, microencapsulated oil was able to reduce between 38 and 100% the growth of species from *Circundati* section and around of 29% the isolated belonging to *Candidi* section.

3.5. Effect on germination power

Table 5 shows the percentage of GP of peanut seeds treated with the boldo EO formulation compared to the untreated control, during a storage period of 114 days. According to ANOVA, GP was significantly affected ($p > 0.01$) by fungicide seed treatment (F: 138.17), level of GP (F: 80.06), fungicide*treatment (F: 34.01) interaction and microcapsule treatment (F: 33.12).

Boldo EO formulation reduced between 39.2 and 48.6% the percentage of germinated seeds with fungicide treatment (F) at 30 days. This reduction became significant ($p < 0.001$) at the end of storage period (114 days) when PG values were between 1 and 6% for seeds in contact with microencapsulated boldo oil.

Seeds without fungicide presented very low GP; both treated and untreated with the boldo EO formulation.

4. Discussion

Encapsulation conditions such as gelatin bloom, crosslinking agent and reticulation time, were investigated and optimized by using D-optimal experimental design to microencapsulated boldo EO by complex coacervation. Optimal encapsulation conditions were found to be: 240 gelatin bloom and formaldehyde 10 min. The microcapsules prepared under these optimal conditions had an average size of 5.67 μm and EE of 94.38%. Both parameters were similar to those obtained by Qv et al. (2011) when lutein was microencapsulated by complex coacervation. Low levels (1.1×10^{-4} -1.5×10^{-3}%) were released from microcapsules after 10–12 days of storage. While, in a previous study about 36.5% of pure boldo EO was recovered from peanut kernels at 11 days of incubation (Passone and Etcheverry, 2014). Thus, it is demonstrated the ability of the microcapsules to delay the boldo oil release. This behavior was not affected by storage temperature (4–25 °C). Peng et al. (2014) found that the stability of the microencapsulated mustard (*Sinapis alba*) seed EO decreased with increasing temperature, when compared microcapsules stored at 25 and 40 °C. The authors attributed this results to the higher temperature was associated with the glass transition temperature of complex coacervation microcapsules. Therefore, although the temperature is important for the storage of heat sensitive materials such as EO, the storage at room temperature would be sufficient to maintain the stability of the boldo oil formulation, without the need to be refrigerated. However, the release rate of boldo EO from microcapsules was affected by the peanut aqueous condition. Higher levels of boldo EO were observed outside of microcapsules when peanut kernels were conditioned at 0.95 a_W. Similarly, Peng et al. (2014) and Qv et al. (2011) reported that lutein and mustard (*Sinapsis alba*) seed EO microcapsules performed by complex coacervation should be preserved under low relative humidity conditions. Relative humidity is considered to be a significant factor affecting the storage stability of EOs. Both gelatin and gum arabic are hydrophilic and highly hygroscopic polymers. When microcapsules are exposed to an environmental humidity, its wall material could form a barrier to protect the EO from water erosion. With the invasion of moisture, the globular structure of the microcapsules will be completely destroyed in a gradual way (Peng et al., 2014).

Table 4
Fungal genera present in peanut seeds treated with boldo EO formulation and control.

	Control				Boldo EO			
	MGP		LGP		MGP		LGP	
Genus	F	WF	F	WF	F	WF	F	WF
Ascomycetes								
Byssochlamys	0.0 ± 0.0	6.3 ± 21.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.8 ± 16.6	0.0 ± 0.0	0.0 ± 0.0
Eurotium	0.0 ± 0.0	43.8 ± 143.9	0.0 ± 0.0	16.2 ± 47.9	0.0 ± 0.0	4.2 ± 14.4	2.1 ± 7.2	13.9 ± 48.1
Deuteromycetes								
Alternaria	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.3 ± 12.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Aspergillus								
Candidi	0.0 ± 0.0	62.5 ± 125.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12.5 ± 25.0	0.0 ± 0.0	0.0 ± 0.0
Circundati	61.5 ± 112.2	37.5 ± 87.6	0.0 ± 0.0	0.0 ± 0.0	20.8 ± 58.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Flavi	59.0 ± 209.2	72.6 ± 339.9	1.0 ± 5.1	6.9 ± 34.0	48.6 ± 205.5	74.7 ± 339.6	0.0 ± 0.0	6.9 ± 34.0
Nidulantes	62.5 ± 176.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	62.5 ± 176.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Restricti	3.1 ± 8.8	0.0 ± 0.0	3.1 ± 8.8	0.0 ± 0.0	6.3 ± 17.7	0.3 ± 0.9	0.0 ± 0.0	0.0 ± 0.0
Cladosporium	6.3 ± 12.5	0.0 ± 0.0	0.0 ± 0.0	68.8 ± 137.5	0.0 ± 0.0	6.3 ± 12.5	0.0 ± 0.0	0.0 ± 0.0
Fusarium	0.0 ± 0.0	6.3 ± 12.5	0.0 ± 0.0	41.7 ± 83.3	0.0 ± 0.0	131.3 ± 262.5	0.0 ± 0.0	41.7 ± 83.3
Metarhizum	62.5 ± 125.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Paecilomyces	0.0 ± 0.0	20.8 ± 72.2	60.4 ± 209.3	0.0 ± 0.0	2.1 ± 7.2	10.4 ± 24.9	0.0 ± 0.0	0.0 ± 0.0
Penicillium								
Aspergilloides	284.4 ± 1124.2	1.6 ± 6.3	1.0 ± 4.2	34.4 ± 124.5	281.3 ± 1125.0	1.7 ± 6.5	1.0 ± 4.2	0.0 ± 0.0
Citrina	143.2 ± 338.1	9.3 ± 37.5*	11.5 ± 41.6	333.3 ± 1206.5	137.0 ± 339.4	9.3 ± 37.5*	11.5 ± 41.6	333.3 ± 1206.5
Divaricatum	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	18.8 ± 37.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Furcatum	0.0 ± 0.0	0.0 ± 0.0	106.3 ± 212.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Penicillium	0.3 ± 1.6*	4.8 ± 25.5*	273.9 ± 1016.4	477.6 ± 1112.1	815.1 ± 3204.0	8.0 ± 31.2	77.0 ± 205.6	112.8 ± 347.4
Simplicia	173.4 ± 521.7	668.8 ± 2575.8	2.1 ± 8.3	0.0 ± 0.0	125.0 ± 500.0	1.6 ± 6.3	3.6 ± 10.1	0.0 ± 0.0
Verticillum	250.0 ± 500.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Levaduras	0.0 ± 0.0	0.0 ± 0.0	12.5 ± 25.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Unidentified morphologically	25.0 ± 28.9	797.9 ± 789.3	310.4 ± 448.3	2437.5 ± 4875.0	0.0 ± 0.0	489.2 ± 794.0	47.9 ± 80.0	12.5 ± 4.4

*(x 10^4). a.Genres and sections according to Samson and Frisvad (2004), Samson et al. (2010), Pitt (2000), Houbraken et al. (2011, 2014), Visagie et al. (2014a, 2014b).
MGP: medium germinating power. LGP: low germinating power. W: with fungicide. WF: without fungicide.

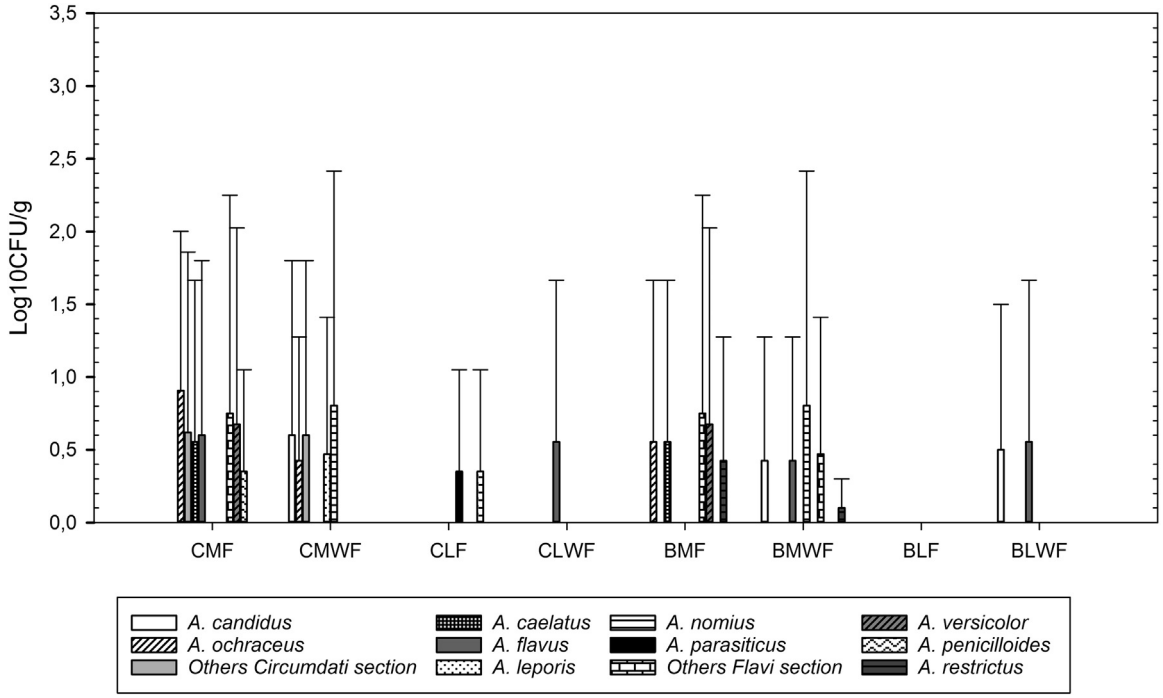

Fig. 5. Effect of boldo EO formulation on *Aspergillus* species.

In this work, further characterization of the boldo EO microcapsules, the ability to control fungal pathogens of peanut seeds in microcosm was also assessed. Although in this work the effect of empty microcapsules as control was not assayed, in a recent study this treatment applied to peanut kernels produced *Aspergillus* section *Flavi* growth reductions in the order of 50–100% compared to the control without capsules, when kernels were conditioned at 0.83 a_W. Nevertheless, when peanut were conditioned at the a_W that represent a high risk of fungal contamination (0.95), *Aspergillus* development was not affected by the presence of formaldehyde in the capsules (García et al., 2016). Agu and Palmer (1999) revealed that low doses (0.1%) of formaldehyde had a good antifungal effect

Table 5
Effect of boldo EO formulation on seeds germination (%).

GP	Fungicide	Treatment	GP (%)			
			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
		Boldo	24.67 ± 2.83c	12.00 ± 0.00b	15.00 ± 1.41b	1.00 ± 1.41a
	WF	Control	5.34 ± 1.89a	4.00 ± 2.83a	0.00 ± 0.00a	1.00 ± 1.41a
		Boldo	5.34 ± 1.89ab	10.00 ± 0.00b	1.00 ± 1.41a	6.00 ± 2.83ab
Medium	F	Control	43.34 ± 4.72a	56.00 ± 19.80a	55.00 ± 1.41a	51.00 ± 12.7a
		Boldo	43.34 ± 4.72b	17.00 ± 1.41ab	29.00 ± 9.90ab	6.00 ± 5.66a
	WF	Control	12.00 ± 1.88a	11.00 ± 7.07a	20.00 ± 5.66a	17.00 ± 4.24a
		Boldo	12.00 ± 1.88ab	4.00 ± 0.00a	28.00 ± 5.66b	5.00 ± 7.07a

Data with different letters for each treatment are significantly different according to LSD test. ($p > 0.01$).

of sorghum seeds. Therefore, formaldehyde used as crosslinking agent in coacervation process could act as antifungal together with the boldo EO.

The boldo oil formulation applied at the doses of 3000 ppm reduced between 31.5–42.5% fungal developments on peanut seeds compared to the control treatments at the end of the storage period (114 d). Thus, inhibitory activity of the boldo formulation on peanut seed fungal pathogens was higher than that produced by conventional fungicide. In previous studies, complete inhibition of *Aspergillus* section *Flavi* was caused by pure boldo EO at 3000 ppm on irradiated peanut kernels conditioned at different a_W (0.93, 0.95 and 0.98) (Passone and Etcheverry, 2014). The decrease of the antifungal effect may be due to the presence of other fungal genus besides *Aspergillus Flavi* section in natural peanut kernels. In fact, most species that developed on seeds belonged to *Penicillium* genus.

Moreover, the boldo EO formulation had an inhibitory effect on peanut seed germination. *In vitro* studies suggested that boldo EO has phytotoxic effects on *Amaranthus hybridus* and *Portulaca oleracea,* and this activity correlated with its high content of oxygenated monoterpenes (Verdeguer et al., 2011). While, Betancur et al. (2010) found that boldo EO did not affected significantly the germination of maize seeds.

5. Conclusions

The results of this study showed that the complex coacervation is a suitable method to entrapping boldo EO with high encapsulation efficiency, allowing controlled release. The formulation reduced the incidence of mycoflora on peanut seeds during a storage period of 114 days. However, an adverse effect on seed germination was produce by the formulation compounds released in the surrounding atmosphere of peanut seeds. To avoid this effect, the implementation of boldo EO formulation on stored shell peanut will be evaluated in future studies.

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