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Polycaprolactone microcapsules containing citric acid and naringin for plant growth and sustainable agriculture: physico-chemical properties and release behavior

Adriana Cesari¹, Mónica V. Loureiro², Mário Vale², E. Inés Yslas³, Marta Dardanelli¹, Ana C. Marques^{2*}

¹ INBIAS, Instituto de Biotecnología Ambiental y Salud, CONICET, Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Ruta Nacional 36, Km 601, Córdoba, Argentina.

² CERENA, Departamento de Engenharia Química, Instituto Superior Técnico, Universidade de Lisboa, Avenida Rovisco Pais, 1049-001 Lisboa, Portugal.

³ IITEMA, Instituto de Investigaciones en Tecnologías Energéticas y Materiales Avanzados, CONICET, Departamento de Biología Molecular, Universidad Nacional de Río Cuarto, Ruta Nacional 36, Km 601, Córdoba, Argentina.

Abstract

Plant growth promoting rhizobacteria (PGPR) is an alternative to chemical fertilizers for sustainable, environment friendly agriculture. There is a need to develop strategies to potentiate the interaction between rhizobacteria and plants. Flavonoids and organic acids (components of root exudates) play specific beneficial roles as carbon sources and signal molecules in the plant – rhizobacteria interactions. The goal of this work is to encapsulate signal molecules, namely citric acid and naringin, an organic acid and a flavonoid, respectively, by a biodegradable polymer, polycaprolactone (PCL), in order to maintain the stability and activity of those signal molecules and enable their slow or controlled release

over a selected period of time, according to the needs of the plants. This approach is expected to potentiate food crops, namely peanut crop, in adverse environmental conditions (water deficit), by promoting the beneficial interaction between the peanut plant (*A. hypogaea*) and rhizobacteria. The microcapsules (MCs) are obtained by an emulsion process combined with solvent evaporation technique and are characterized by scanning electron microscopy, thermogravimetry and Fourier transformed infrared spectroscopy. The kinetics of *in vitro* release of encapsulated molecules, in a period where the uptake of the compound in plants can occur, is studied. The encapsulation synthesis parameters that lead to the best encapsulation process yield and efficiency, as well as to the best final performance in terms of release, are identified. The effect of pH and molecular weight of PCL is found to mediate the release properties of the molecules for different types of soil. PCL 45000 Mw dissolved at 16% in dichloromethane leads to an encapsulation efficiency of 75% and the resulting MCs containing naringin exhibit a slow release profile for 30 days, unmodified by pH, enabling their use in soils of different characteristics. This research makes possible the manufacturing of smart materials for sustainable agriculture practices.

Keywords

Polycaprolactone; Microencapsulation; Naringin; Citric acid; Controlled release; Plant growth promoting rhizobacteria

1. Introduction

Due to the rapid growth of the world population, modern agriculture plays a critical role in meeting the demand for food and other agro-products. Fertilizers and water are the most important elements in agricultural production. The most important characteristic of commercial chemical fertilizers is instant dissolution, however because the nutrient release rate per unit of time is often much higher than the adsorption rate of crops, there is overdosing with subsequent negative effects as soil compaction and contamination and the decrease in crop yields (Pang et al., 2018). As an alternative to chemical fertilizers, the development and use of microbial inoculants based on plant growth promoter bacteria (PGPR) has been increasing worldwide (Askary et al., 2009; Malusa and Vassilev, 2014; Cesari et al., 2019). This trend complies indeed with the directives for sustainable agriculture practices and is aligned with one of the key Sustainable Development Goals (SDG) set by the United Nations, which aims by 2030 to “end hunger, achieve food security and improved nutrition and promote sustainable agriculture”.

In order to reduce environmental problems and the negative impact caused by conventional chemical fertilizers, encapsulation of fertilizers for slow or even controlled release has been proposed to enable continuous release of nutrients throughout the growth season (Tolescu *et al.*, 2014; Chen L. *et al.*, 2008; Chen S. *et al.*, 2018; Chen J. *et al.*, 2018). Microcapsules (MCs) are mainly used as carrier systems for the storage and protection of functional compounds or the slow or controlled release of active compounds (Rathore *et al.*, 2013; Loureiro *et al.*, 2017a, b, 2019; Attaei *et al.*, 2018), which makes them potential smart materials for agriculture applications.

Biodegradable polyesters such as poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA) and polycaprolactone (PCL) have been widely used as carriers in controlled-release

delivery systems. The degradation of these polyesters involves a bulk erosion process, which can accelerate the dilution and release of the drug (Makadia *et al.*, 2011). PCL is one of the biodegradable polyesters which have attracted attention to be used in controlled drug delivery bioapplications due to its lack of toxicity and low cost when compared to other biodegradable polyesters. One example is the development of microspheres, matrix-type, containing quercetin or triprolidine hydrochloride incorporated in the PCL matrix (Natarajan *et al.*, 2011; Sudhakar *et al.*, 2014).

Arachis hypogaea (peanut plant) is a vegetal with high nutritional value, the sixth most important source of oil and the third most important source of vegetable protein in the world (Mahatma *et al.*, 2016). In Argentina, about 90% of peanut plantation takes place in Córdoba province which are mainly exported to European Union, Indonesia, Canada, etc. (INTA, 2018). Taking into account the agronomic importance of peanut crop it is important to develop sustainable strategies to increase its production. Plant root exudates (RE) encompass primary and secondary metabolites (amino acids, sugars, nucleotides, organic acids, fatty acids and flavonoids) that lead to acidification of the rhizosphere (from pH 7 to pH 4 -5) and are necessary for establishing a network of interactions between the plant roots and the PGPR, to aid the plant growth process (Bais *et al.*, 2006, Haichar *et al.*, 2014; Yuang *et al.*, 2015, Cesari *et al.*, 2019a,b). Flavonoids and organic acids present in RE play specific roles as carbon sources and molecular signals in plant-microbe interactions (Kloss *et al.*, 1984; Jones, 1998). The concentration of flavonoids in the rhizosphere is between 2 μM and 50 μM (Fabra *et al.*, 2010; Sugiyama *et al.*, 2017; Cesari *et al.*, 2019), while the amount of organic acids is 100-800 $\text{mg}\cdot\text{g}^{-1}$ per root (Song *et al.*, 2012). Flavonoids, as naringin, act as inducers of several *nod* genes in the *Rhizobium* spp., necessary to carry out the initial event of the symbiosis and an effective fixation of the atmospheric nitrogen (Amallesh *et al.*, 2011;

Fox et al., 2011; Falcone Ferreyra et al., 2012). Citric acid and malic acid are the main organic acids detected in RE and have been reported as chemoattractant agents for rhizobacteria and inorganic phosphorus solubilizers (Wong, 2004; Kamilova et al., 2006).

In the following of our previous work (Cesari et al., 2019a,b), the present paper deals with the protection and controlled release of signal molecules, typical root exudates, such as the flavonoid naringin and the organic acid, citric acid, through their encapsulation by a biodegradable polymer (PCL). This effort is aimed at boosting the plant (e.g. peanut) growth, by promoting the beneficial interaction between the plant and PGPR and, therefore, replacing the conventional chemical fertilizers by molecules typically present in RE. In this work we develop MCs whose content release responds to pH through a slow degradation of the PCL shell resulting in a controlled release of the encapsulated compound during rhizosphere acidification. These MCs are prepared by the solvent evaporation method combined with a water-in-oil-in-water (w/o/w) emulsion system, to generate a matrix or shell that protects naringin and citric acid from the environmental conditions and allow their controlled release, according to the needs of the plant. It is a physical, straightforward and flexible method for MCs preparation, and the solvent employed can be easily recovered and recycled. Constant stirring speed and temperature, in combination with a constant evaporation rate are the main keys to ensure a good reproducibility of the syntheses (Gonzalez *et al.*, 2015; Iqbal *et al.*, 2015; Arshadi, 1991; Li *et al.*, 2008). The effect of the concentration and molecular weight (Mw) of the PCL used for the MCs synthesis, as well as the water and oil phases proportion on the encapsulation performance were studied. This is accomplished by a physical-chemical characterization of the MCs to evaluate the effects of pH and RE on the encapsulated molecules release kinetics.

Finally, it should be stressed that not many articles where the encapsulation of sustainable biofertilizers is reported, are found in the literature (Li et al., 2017; Tu et al., 2016; Tu et al., 2015; Wu et al., 2014). Microencapsulation of bacterial cells (bacterial fertilizers) was reported using a mixture of sodium alginate and maltodextrin or polybutylene succinate and starch as a cover material (Campos et al., 2014; Wu, 2008); plant growth regulators, such as *a*-naphthalene acetate, were loaded into double-layered inorganic matrices (Hussein et al., 2002); bioflavonoids, such as hesperidin, were encapsulated within alginate microparticles (Tsirigotis-Maniecka et al., 2017). None of them regards the microencapsulation of active species, by biodegradable polymers, namely PCL, achieved by means of a straightforward method based on microemulsion/solvent evaporation, with the envisaged target of this work. Our work lays the groundwork for the application of molecules of interest for agricultural use, by means of microencapsulation approaches.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL) (80000 Mw, 45000 Mw and 30000 Mw), citric acid and naringin were obtained from SIGMA-Aldrich. Dichloromethane (DCM) was obtained from VWR chemicals. The emulsifier Gum Arabic was purchased from LabChem and poly vinyl alcohol (PVA) (88% hydrolyzed, 22000 Mw) was obtained from Sigma-Aldrich. In this study, PVA was used as the emulsion stabilizer, together with Gum Arabic. DCM was used as the organic solvent for PCL and citric acid and naringin were the molecules to be encapsulated. All the chemicals were used as received, without further purification.

2.2. Preparation of PCL microcapsules

The PCL MCs loaded with citric acid or naringin were prepared by combination of a water-in-oil-in-water ($W_1/O/W_2$) double emulsion system and solvent evaporation technique. The W_1 phase, consisting of 7.2 wt% of the total emulsion system, was composed of naringin at 1 wt% or 5 wt% in water, for the case of MCs containing naringin, or citric acid at 80 wt% in water or as solid drug, for the case of MCs containing citric acid. A solution of PCL in DCM was used as the organic phase of the emulsion system, consisting of 26 wt% of the total emulsion system. The syntheses were performed using PCL with different Mw of 30000, 45000 and 80000 g/mol in order to assess the effect of the PCL Mw on the MCs' formation and release behavior in the final application. The W_2 phase was composed of an aqueous solution of 2 wt% of PVA and 1.5 wt% of arabic gum, both used as emulsion stabilizers. The parameters used in each synthesis are listed in Table 1.

The MCs were prepared by dispersion of the W_1 emulsion phase in the oil phase using a high speed homogenizer (Ultra-Turrax T25) at 10000 rpm for 60 seconds to obtain the first emulsion system (W_1/O). This was then added to the W_2 phase under mechanical stirring, at 500-700 rpm, depending on the PCL Mw. The syntheses were carried out at such selected stirring speed for a certain time (1 to 3 hours) at room temperature (25 °C), until the MCs shell attained enough maturity to tolerate the pressure applied during the filtration procedure. The MCs were then filtrated using a vacuum filtration system, washed three times with distilled water and dried at room temperature for 24 h.

It should be stressed that for each MCs type disclosed in Table 1, three identical and independent syntheses were performed. The temperature in the reactor was kept constant (25 °C) during the syntheses, as well as the stirring speed, which are parameters that have been

reported to contribute to a constant evaporation rate of the employed solvent (Arshadi, 1991, Li *et al.*, 2008, Gonzalez *et al.*, 2015, Iqbal *et al.*, 2015).

[Please insert Table 1 around here]

2.3. Physico-chemical characterization of the PCL Microcapsules

2.3.1. Optical Microscopy

An optical microscope (MSZ 5600, Kruss) was used in order to evaluate the stability of the emulsion, the droplets' size and the MCs' shell maturity during the synthesis procedure, as well as the MCs size. Images of the MCs were captured using an eyepiece ocular lens and analyzed using the software ImageJ (v 1.51p, National Institutes of Health, Bethesda, MD, USA) in order to calculate the individual MCs size and their mean value. After calibrating the scale, a median filter with a radius of 2.0 pixels was applied. The contrast of the images was enhanced, and the MCs were highlighted by a thresholding function. After the image treatments, to remove debris and agglomerates, the data for the size distribution of the MCs was obtained using the Analyze Particles functionality of the software.

2.3.2. Scanning electron microscopy (SEM)

The morphology, size distribution and average shell thickness of the obtained MCs were assessed through SEM, using a JEOL JSM7001F (JEOL, Tokyo, Japan) with a FEG-SEM (Field Emission Gun) scanning electron microscope, operating at 15 kV. The samples were previously coated with a conductive Au/Pd thin film, through sputtering, using a Quorum Technologies sputter coater, model Q150T ES.

2.3.3. Fourier transformed infrared spectroscopy (FTIR)

The relative encapsulation efficiency, as well as the molecular structure of the MCs' shell material were assessed by FTIR spectroscopy in the attenuated total reflectance (ATR) mode. Spectra of the compounds to be encapsulated as well as the MCs' shell constituents were obtained for such purpose. The FTIR equipment used was a PerkinElmer, Spectrum Two, FTIR spectrometer, equipped with a Pike Technologies MIRacle® ATR accessory. The spectra were obtained with 8 cm^{-1} resolution and a data collection of 16 scans.

2.3.4. Thermogravimetric analysis (TGA)

TGA was performed, using a HITACHI STA 7200 Thermal Analysis System equipment, under a controlled nitrogen atmosphere (200 ml/min), at a temperature increase rate of $10\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$, in the range of $30\text{-}600\text{ }^{\circ}\text{C}$. The analysis of the resulting thermograms enabled to quantify the amount of encapsulated compound in the obtained MCs. This technique was also used to corroborate FTIR analysis results.

2.4. Encapsulation process yield and encapsulation efficiency

To calculate the yield of the encapsulation process, the weight of the obtained dried MCs and the weight of all the reagents used for the MCs' manufacture were considered. The encapsulation process yield equation is as follows:

Encapsulation process yield (%)

$$= 100 \times \frac{m(\text{dried MCs})}{m(\text{compound to be encapsulated added to the synthesis}) + m(\text{polymer})}$$

Moreover, the theoretical loading was also calculated, given by:

Theoretical loading (%)

$$= 100 \times \frac{m(\text{compound to be encapsulated added to the synthesis})}{m(\text{compound to be encapsulated added to the synthesis}) + m(\text{polymer})}$$

And, therefore, the encapsulation efficiency (EE) is given by the formula:

Encapsulation efficiency (%)

$$= 100 \times \frac{\text{Experimental loading (\%), obtained by TGA}}{\text{Theoretical loading (\%)}}$$

2.5. Release behavior of the PCL Microcapsules

2.5.1. *In vitro* release studies

In order to study the release behavior of the encapsulated citric acid and naringin, MCs were placed in aqueous solutions at pH 7, 5 and 4, in a proportion of 25 mg of MCs to 1 ml of solution, for 40 days at room temperature (25 °C). In order to evaluate the progress of the cargo release from the MCs, along the time, aliquots of 1 ml were taken at predetermined time intervals and replaced with the same amount of fresh solution. The amount of citric acid and naringin in the collected samples was analyzed by UV spectroscopy (the absorption peak for citric acid is centered at 208-211 nm and that for naringin is at 283 nm) using an UV-Vis spectrophotometer (ATI Unicam, UV2), at 2 nm resolution. Solutions containing citric acid and naringin at known concentrations were used to get a calibration curve for such purpose. As blank measurements, analogous experiments were carried out with solutions containing empty MCs. Triplicates of each MCs dispersion were prepared at different pH values, and in addition the release behavior of the MCs was studied in a RE

environment. The RE was collected from a hydroponic system, in which a peanut plant was grown in a Hoagland solution (Hoagland and Arnon, 1938), as explained in 2.6.

The results were expressed as the cumulative release (in %) of citric acid and naringin, with respect to the amount of encapsulated cargo, being calculated using the following equation:

$$\text{Cumulative release (CR\%)} = (M_t / M_0) \times 100$$

Where M_t is the mass of the released citric acid or naringin in a sample collected at a certain time and M_0 is the initial mass of citric acid or naringin present in the MCs.

Additionally, a mechanism of citric acid and naringin release was proposed taking into consideration the obtained results, by fitting the release profiles with a relevant model, as explained below.

2.5.2. Release mechanism studies

Several types of drug release mechanisms from matrices described by kinetics models have been proposed by several authors (Fernandez et al., 2009). The compound release usually implies water penetration in the matrix, swelling, diffusion of the dissolved drug, matrix degradation and saturation of the matrix pores with release medium. However, it is worth to mention that the release mechanism of a drug depends on the dosage, pH, nature of the drug and polymer used.

In this present study, the mechanism of citric acid and naringin release from the PCL MCs was investigated using a semi-empirical model, known as the power law or the Peppas model (Peppas, 1985) since it takes into account the Fickian diffusion phenomenon and the relaxation phenomenon of the polymer chains:

$$\frac{M_t}{M_\infty} = k \cdot t^n$$

where M_t and M_∞ represent the amount of nutrient released at a time t and at equilibrium, respectively, k is the constant characteristic of the compound-polymer system, and n is the diffusion exponent characteristic of the release mechanism, which allows to determine the phenomena present during the release process (Peppas, 1985; Costa and Sousa Lobo, 2001; Siepmann and Göpferich, 2001; Iborra, 2008). When $n = 0.5$ the release of the compound follows a Fickian type diffusion mechanism. An anomalous or non-Fickian diffusion occurs when $0.5 > n < 1$. A *quasi*-Fickian diffusion process occurs for $n < 0.5$, in cases where the matrix is a porous material and, therefore, there is a combined partial diffusion either through a swollen matrix and the pores filled with water. Values of $n < 0.5$ denote the existence of another process simultaneous to the diffusion. In the case of $n = 1$ the kinetics of the release system is zero order, being the release process controlled by the relaxation of the polymer chains with the diffusion occurring at a constant speed, if the geometry of the system does not change during the release process.

2.6. Peanut root exudate collection

To collect the peanut RE, a germinated peanut seed was aseptically transferred to a hydroponic system consisting of a glass tube containing 30 ml of a Hoagland (pH 6.5) nutrient solution (Hoagland and Arnon, 1938). It should be stressed that the seed does not fall to the bottom, since it is held by a wire device. The germinated peanut seeds were incubated aseptically for 7 days in a growth chamber subjected to a photoperiod of 16 h of light exposure at 24 °C alternating with 8 h of darkness at 20 °C (Dardanelli et al., 2008b). On the seventh day, the plants were removed from the tubes. Sterile samples were kept at 4

°C and the exudates collected and centrifuged at 10000 rpm for 20 min to remove root debris, and stored at -20 °C until their use.

3. Results and discussion

3.1. Encapsulation process yield and morphology of the microcapsules

In this work, several studies were conducted in order to determine the optimal synthesis conditions for the MCs production, as listed in Table 1, namely the effect of PCL Mw and concentration on the encapsulation yield and MCs response to relevant pH in the final application. The encapsulation process yields as well as the MCs' size distribution are given in Table 2. It should be stressed that the MCs obtained from the three identical and independent syntheses, carried out for each type of MCs, displayed the same morphology and a relatively broad size distribution, which is typical of this technique, as exhibited in Table 2. Identical encapsulation process yields were achieved, shown by the low standard deviation (SD) values.

[Please insert Table 2 around here]

In the present work, the higher yields were obtained with the MCs produced using PCL of 45000 Mw and 30000 Mw at 16% in DCM as the oil phase of the emulsion system, for both the citric acid and naringin encapsulation. Encapsulation yields of up to ca. 73% were achieved, for naringin. It should be stressed that the synthesis parameters have been optimized for lower Mw PCL grades, and indeed, it was observed that the lowest encapsulation yield was obtained using the PLC with longer polymeric chains (80000 Mw)

for both the encapsulation of citric acid and naringin, since the same reaction parameters were employed for the encapsulation with PCL 80000 Mw. A significant increase of the O phase viscosity was observed when using PCL 80000 Mw, which was responsible for a destabilization of the emulsion. Also, this high Mw PCL typically experiences solubility issues, so that the lower yields obtained for syntheses employing higher Mw PCL are probably due to losses of PCL, which is not involved in the shell formation. A trial to improve the process yield with PCL 80000 Mw was carried out, by further diluting PCL, or/and by increasing the mechanical stirring speed and, indeed, this has resulted in an increase of the process yield from 3 to 13.6% and from 1.4 to 31%, for citric acid and naringin, respectively. These were the samples that were selected for the thermogravimetric studies.

As shown in Table 2, the size of the MCs was found to be within the range of 99 to 726 μm . Further analysis of the results shows that the MCs' size depends either on the Mw of the PCL used, as well as on the compound to be encapsulated, which might be related to differences in the viscosity and density of the W_1 and O phases. Also, despite the significant difference in encapsulation yield among M80(16)C(80) and M80(12)C(80), they present very similar sizes. The same for M80(16)N(5) and M80(12)N(5). In what regards naringin encapsulation, the use of PCL with 80000 Mw as shell material led to bigger MCs, with the M80(16)N(5) having the largest size, followed by M80(12)N(5), which was expected since the latter ones were prepared with less concentrated PCL and, therefore, with a less viscous O phase compared to the former ones. In this case, the MCs size increases with the increase of the PCL Mw. This is in agreement, for instance, with the work of Shin et al., (2012) where MCs containing imidazole are prepared with PCL of 80000 Mw, 65000 Mw and 14000 Mw. They report that the obtained MCs had almost uniform shapes and the size

increased with the increasing of the PCL Mw. Although not so notorious, it was also observed that the more concentrated was the W_1 phase, i.e. the solution of the compound to be encapsulated, the larger the size of the obtained MCs. In particular, the PCL MCs containing citric acid exhibited a larger dimension when citric acid is encapsulated in the pure form (M45(16)C(100)) than when diluted in a concentration of 80wt% (M45(16)C(80)). In this case, PCL with 45000 Mw was the one to result in larger MCs.

It can be observed that, when changing the encapsulated compound and its percentage, as well as the PCL Mw, different viscosities will be obtained for both the phases of the W_1/O emulsion system, which could lead to destabilization resulting in some coalescence and consequently bigger size MCs are formed. Also, optimization of the mechanical stirring speed can be used to obtain a more homogeneous MCs' size and higher encapsulation yields.

Fig. 1 shows the SEM photomicrographs of the obtained MCs, at different degrees of magnification. The SEM analysis enabled to assess the MCs morphology, typology and aggregation degree. Some of the MCs were crushed on purpose on the preparation for the SEM analysis in order to make it possible to conclude about their morphology and determine their shell thickness. All the MCs were found to have a spherical shape, a core shell morphology and no significant aggregation. On the other hand, most of the samples were found to exhibit some holes on the surface, being less notorious in samples M80(12)C(80) and M45(16)N(1). This phenomenon can be attributed either to the evaporation of the W_1 phase solvent, as to the diffusion of water between W_1 and W_2 phases during the MCs synthesis, through the polymeric membrane (pre-formed shell) while it is not sufficiently solid (Weidenauer *et al.*, 2003; Zhou *et al.*, 2011).

[Please insert Fig. 1 around here]

It is possible to observe that the MCs synthesized using PCL of 30000 Mw are the ones presenting more and bigger surface holes. Indeed, the PCL of 30000 Mw offers less resistance to the diffusion of water, in comparison with PCL of higher Mw. This latter polymer grade, of longer polymeric chains, tends to exhibit higher mechanical strength than the smaller polymer chain grades. The MCs synthesized using PCL of 45000 Mw (Fig. 1C-F; M-P) presented a smoother surface when compared to the MCs obtained using PCL of 80000 Mw, while the MCs formed with PCL 80000 Mw (Fig. 1G-J, Q-T) present a thicker shell. It should also be noted, for samples M30(16)N(5) and M45(16)N(5), the presence of naringin in its crystalline form (Fig. 1L-P, respectively). Naringin has been reported to crystallize from water as an octa-hydrate molecule with a melting temperature at 83 °C and is characteristic for the high water uptake. It crystallizes as needles which are usually found agglomerated in a rosette pattern (Hendrickson *et al.*, 1956). Finally, there is also some evidence for the occurrence of naringin crystals embedded within the PCL forming the shell of the MCs, namely for sample M80(16)N(5), as shown in Fig.1T.

3.2. Chemical structure of the microcapsules

FTIR spectroscopy was performed to assess the presence of characteristic groups in the MCs, providing information on the composition of the matrix (shell) and the presence of citric acid or naringin in the MCs. Fig. 2 shows the FTIR spectra of PCL, citric acid, naringin and the selected MCs.

[Please insert Fig. 2 around here]

The strong characteristic peak at 1720 cm^{-1} present in the FTIR spectrum of PCL, is ascribed to the carbonyl C=O stretching vibration. It is also possible to identify the symmetric COC stretching vibration peak at 1170 cm^{-1} . The spectrum of citric acid exhibit typical bands of organic acids, such as C=O stretching, C-O stretching, OH bending and C-H stretching (Moreira and Santos, 2005; Sharkawy *et al.*, 2017). The FTIR spectra of all the citric acid MCs (Fig. 2.A-B) show an intense peak at 1720 cm^{-1} , ascribed to the stretching of the PCL carbonyl group, which confirms its presence in the MCs' shell structure. However, this peak exhibits a slight shoulder at lower and higher frequency, which comes from the characteristic peaks of the citric acid spectrum in this region of the spectrum, evidencing its encapsulation by the PCL structure. Additionally, a wide band at $3250\text{-}3750\text{ cm}^{-1}$ can be observed in these spectra, which might correspond not only to the OH groups from the water used in the citric acid solution, but also to the intramolecular OH groups belonging to the citric acid. It should be stressed that the MCs were only dried at RT.

Fig. 2C-D shows the FTIR spectra of PCL, naringin and selected MCs. A broad and clear band between $3000\text{ and }3500\text{ cm}^{-1}$ is displayed in the naringin spectrum, being assigned to –OH groups stretching vibration (Sahiner *et al.*, 2018), probably due to its presence in the benzene rings as well as in the glycoside structure of naringin. In what regards the naringin MCs, it is also possible to confirm the presence of the carbonyl peak at 1720 cm^{-1} , revealing that their shell is composed by PCL. The broad band around 3400 cm^{-1} could also be derived from the aqueous solution employed in the synthesis. In addition, peaks ascribed to carbonyl (C=O) bonds and C-C bonds from benzene rings can be observed at 1640 cm^{-1} , and at 1500 cm^{-1} , respectively, which further reveals the encapsulation of naringin in these MCs. The difference observed in the carbonyl peak wavenumber in the PCL and naringin spectrum can

be correlated to the type of carbons the carbonyl is attached to, which in the case of naringin is a saturated carbon ring (Smith, 2017; Sahiner et al., 2018). Fig. 2B-D show a magnified image of a relevant wavenumber range, where the fingerprint of citric acid and naringin in the MCs spectra is clearly observed, suggesting their effective encapsulation.

3.3. Thermogravimetric and encapsulation efficiency of the microcapsules

The thermograms of the PCL, encapsulated compounds and the MCs are shown in Fig. 3.A-B.

[Please insert Fig. 3 around here]

According to the thermogram in the Fig. 3.A the citric acid and the PCL were completely degraded in a single step, the citric acid from 200 to 300 °C and the PCL from 340 to 450 °C. However, the thermograms obtained from MCs exhibit two to three distinct degradation steps. The step of weight loss in the range of 30 to 100°C, not observable for the PCL nor the citric acid, might be correlated with the presence of water, in particular from the aqueous citric acid solution used in the MCs preparation. In fact, the MCs that do not exhibit this low temperature degradation step are the ones where citric acid is not diluted (M45(16)C(100)) and the ones prepared with PCL 30000 Mw, which, in this latter case, might be due to the thinner and more porous PCL shell, that allows a more efficient drying of the MCs core. The presence of water, at 7.7 wt% and 5.8 wt%, was detected in the MCs M45(16)C(80) and M80(12)C(80), respectively. The degradation peaks between ca. 160 to 350 °C and ca. 350 to 450 °C can be ascribed to the degradation of citric acid and PCL, respectively. Therefore, since these two degradation phenomena are quite distinct in terms of temperature range, it is

possible to estimate the content of citric acid encapsulated in the MCs, i.e. the citric acid encapsulation efficiency. Samples M30(16)C(80), M45(16)C(100), M45(16)C(80) and M80(12)C(80) present a content of encapsulated citric acid of ca. 4 wt%, 9 wt%, 15 wt% and 25 wt%, respectively, of the total MCs weight, which suggests that the higher the PCL Mw, the more efficient the citric acid encapsulation. In fact, Table 3 shows this trend in terms of encapsulation efficiency values. Also, it can be concluded that the use of an aqueous citric acid solution works better than using non-diluted citric acid (in the powder form). However, it should be stressed that the highest encapsulation efficiency achieved regarding the encapsulation of citric acid was 38.5%, for M80(12)C(80). It is believed that this value might be further improved, by carrying out a synthesis optimization procedure.

[Please insert Table 3 around here]

The low encapsulation efficiency values found for citric acid loaded MCs might be due to the holes found in the MCs' shell. In fact, the SEM photomicrographs of M80(12)C(80) show that these MCs are the ones displaying less holes.

In what regards, the thermograms of the MCs prepared with naringin (Fig. 3B), three steps of weight loss can be detected, the first between 30-100°C, which might be due to the presence of water in the MCs, and two final steps between 254-350 °C and 350-450°C, correlated with the degradation of naringin and PCL, respectively. Naringin thermogram shows a slight decrease in mass (6 wt%) below 200 °C and shows no weight loss between 200-250 °C. When naringin is subject to temperatures above 250 °C, a strong progressive weight loss can be observed, with the sample losing a total of 65% of its total mass by 600 °C. As for the MCs with citric acid, those prepared with naringin also exhibit three steps of weight loss during the same temperature range, being the first between 30-100°C, probably

due to the presence of OH groups and adsorbed moisture, and two higher temperature steps between 254-350 °C and 350-450°C, correlated with the degradation of naringin and PCL, respectively (Fig. 3B). The amount of encapsulated naringin is, therefore, estimated to be ca. 3 wt%, 5 wt% and 6 wt% for the MCs M30(16)N(5), M80(12)N(5) and M45(16)N(5), respectively (Table 3). The lower amount of encapsulated compound in the MCs containing naringin, in comparison with the MCs containing citric acid, is in part due to the lower amount of naringin used during the synthesis, due to its low solubility in water. However, it should be noted that the naringin containing MCs were the ones with the best overall encapsulation efficiency (Table 3), with values in the range of 38 and 75.6%, with the best ones being M45(16)N(5), followed by M80(12)N(5).

3.4. *In Vitro* Release Studies

Fig. 4 shows the cumulative release profile of citric acid and naringin from the PCL MCs into the aqueous media at different initial pH values (4 - 5 - 7) and also into *Arachis hypogaea* RE (pH 5).

[Please insert Fig. 4 around here]

It should be noted that the reported data correspond to the average values obtained from three independent release experiments, which are provided in the graphs with the respective SD values. As observed in Fig. 4, the error bars are relatively small, which confirms that the same type of release behavior is achieved for different batches of MCs synthesized following the same procedure.

For M30(16)C(80) MCs, i.e. those prepared with lower Mw PCL, a fast release of citric acid can be observed after 3 days, being faster at pH 4 and 5. The citric acid content was released at 100% after 15 days, both in aqueous solutions and in the RE (Fig. 4A). The release of the total encapsulated content can be attributed to the thinner shell and the surface holes observed for the MCs obtained with PCL of 30000 Mw. In fact, the presence of surface holes can be herein claimed to be an advantage, since the degradation of PCL is known to be slow, being the release of small drugs supposed to be dilution controlled (Sudhakar et al., 2014). A complete release of the total encapsulated material did not occur with the MCs of PLC 45000 and 80000 Mw. In many cases, drug release has been shown to be incomplete, mainly due to the high crystallinity and hydrophobicity of PCL (Shen et al., 2000). The release of citric acid from M45(16)C(80) was pH dependent: 93% at pH 4 after 18 days, 78% at pH 5 after 40 days and 67% at pH 7 after 43 days. When the MCs were placed in RE, the release of citric acid was 35% after 45 days (Fig. 4B). Similar to the above, with the MCs of PCL 80000 Mw the release of citric acid was 70% at pH 4-5 and 28% at pH 7 (Fig. 4C). When the MCs were placed in RE, the release kinetics was similar to that observed in pH 4-5 solutions, although the maximum release was 60%. For the MCs containing citric acid, according to our results, the PCL material composing the MCs' shell exhibited a different response according to the pH of the aqueous solution ($\text{pH } 4 > \text{pH } 5 > \text{pH } 7$), which confirms its pH response capability, enabling a controlled release of the encapsulated compound, as desired. This is a novel and important result for a possible agronomic application, since it is known that the soil zone where the microorganisms PGPR colonize the roots of the plants is the rhizosphere which has an acid pH (around pH 4-5).

Fig. 4 (D-E-F) shows the release profile of naringin from PCL MCs in aqueous solutions at different pH solutions. Unlike that observed for the release of citric acid, the release of

naringin from the MCs was found to be independent on the pH of the medium. MCs M30(16)N(5) showed a quick release after 4 days in the aqueous solutions, with a full release accomplished, similarly to MCs M30(16)C(80). When those MCs were placed in RE, the release of naringin reached 80% after 5 days. As for M45(16)N(5), they showed a gradual release behavior of naringin for 30 days, being slightly higher at pH 4 and 5, than at pH 7. When the MCs were placed in RE the naringin release was 80%, like that observed at pH 7. For M80(12)N(5) the release of naringin was gradual, slow and also independent of the pH of the medium. When these MCs were placed in RE, a 32% of naringin release was observed after 8 days, and then a gradual release was observed reaching the 67% after 45 days. Scarfato *et al.* (2008) reported the release of the flavonoid quercetin from microspheres made from cellulose acetate phthalate and cellulose acetate propionate. It was found in this study that the higher the concentration on cellulose acetate phthalate in the formulation, the higher its viscosity and the lower the quercetin release from the resulting microspheres, namely 25% of release versus 98% at pH 6.8, for low amounts of this reagent. In the present case, the release of naringin from the MCs depends on the Mw of the PCL, being slower and gradual in the MCs of PCL 45000 Mw and PCL 80000 Mw, which clearly evidences the dependence of the MCs' morphology and shell characteristics on the PCL Mw, as referred above. The pH of the medium is herein found to display some effect on the release behavior of naringin, although its effect is not as notorious as in the case of MCs with citric acid. This might be due to the fact that citric acid when released lowers the pH of the aqueous solution (from 7.5 or 4 to 2.5), which leads to a more noticeable pH effect.

3.5. Release mechanism studies

In order to investigate the release mechanism of citric acid and naringin from the PCL MCs in water solution of different pH and in RE environment, data were fitted to *Peppas* equation (described in 2.5.2 section) a method already employed in the study of PCL microparticles containing other types of flavonoids (Lee *et al.* 2007). Results of n , k and r for all the formulations were estimated using the least squares procedure and are listed in Table 4.

[Please insert Table 4 around here]

In our case the n values of different MCs were variable, depending on the PCL Mw, properties of the encapsulated compound and the pH of the solution where the release was made to occur. Values of $n < 0.5$ were achieved for PCL45(16)C(80) and PCL80(12)C(80) at pH 7, 5 and 4 (except for PCL30(16)C(80) at pH 7), indicating that the release of citric acid in the media follows a *quasi*-Fickian diffusion mechanism. Citric acid is herein suggested to partially diffuse through the matrix pores. Upon the contact with the release medium, the citric acid present at the surface of the drug-laden matrix, tends to rapidly dissolve (due to the high solubility of the solute in the release medium) and is released, generating the "burst release". Nevertheless, when the citric acid release was studied in RE, it was found that $n > 0.5$ or even > 1 . Values of n greater than 0.5 are associated with an anomalous (non-Fickian) diffusion mechanism. In the case of PCL45(16)C(80) and PCL80(12)C(80), n displays values above 1. The kinetics in this case corresponds to anomalous non-Fickian diffusion mechanism and regards polymeric matrices in which the swelling of the polymer progresses steadily and very long release times (Costa and Sousa Lobo, 2001; Siepmann and Siepmann, 2008). As shown in Fig. 4 (b and c), the compound encapsulated in the MCs was not fully released when the releasing medium was RE. On the

contrary, it was released from 20 to 40% respectively. This fact can be explained in the following way: as more citric acid is released into the RE, more tendency for the release medium to "saturate", moment at which the release process will end. It should be stressed that RE contains organic acids, so saturation is even faster.

For MCs loaded with naringin, the value of n was found to increase as the Mw of PCL increases. PCL30(16)N(5) MCs display values of n below 0.5, which indicates that naringin diffuses partially through a porous matrix (*quasi*-Fickian diffusion mechanism). For PCL45(16)N(5) MCs, the values of n were close to 0.5, indicating a Fickian-type diffusion mechanism. While for PCL80(12)N(5) the values were $n > 1$ for different pH and RE, indicating anomalous non-Fickian diffusion mechanism. In these cases, a full release of the total encapsulated compound is not possible, since the release medium "saturates" quickly. Values of $n > 1$ have also been reported for other PCL capsules (Sahoo et al., 2010; Kulkarni et al., 1999).

4. Conclusions

Successful encapsulation of molecules involved in the *Arachis hypogaea*-PGPR interaction, such as citric acid and naringin, was carried out using the biodegradable polymer PCL, by a double microemulsion $W_1/O/W_2$ technique combined with the solvent evaporation method. To our knowledge, this study is the first to report PCL's MCs containing citric acid and naringin for a slow or controlled release in agronomic applications.

Our results demonstrate that PCL 45000 Mw is shown to be an efficient vehicle for the encapsulation of signal molecules involved in the plant-PGPR interaction. The use of PCL 45000 Mw (diluted at 16% in DCM) for the synthesis of the MCs containing citric acid or naringin, can be a potential solution for the current need regarding sustainable agro-

industrial practices. Citric acid was found to be more efficiently encapsulated when diluted at 80 % in water. MCs loaded with citric acid presented a slow release during 45 days and the release kinetics is pH-responsive (pH 4 > pH 5 > pH 7), independent of the PCL Mw, which means that a controlled release might be achieved with these MCs. When the release in RE was analyzed, it turned out to be more gradual, which could eventually lead to a more sustained delivery of signal molecules to the ground in a real application. On the other hand, MCs containing naringin present a slow release for 30 days, unmodified by pH, which indicates that it could be used in soils of different characteristics, in an indiscriminate manner, and facilitate the continuous supply (slow release) of nutrients to the plants.

The release kinetics of the compounds could be fitted by the *Peppas* model. As a result, the release of citric acid, except in some cases, is found to occur by a *quasi*-Fickian diffusion mechanism, while the release of naringin is closer to occur by a Fickian type diffusion mechanism.

The achieved results confirm the initial assumptions of this research and create a real possibility of manufacturing intelligent materials for sustainable agriculture, since they are able to deliver signal molecules involved in plant-microorganism interaction, “when and where needed”. This work will be followed by applying these MCs to peanut seeds and studying their effect on plant-PGPR events and plant growth parameters.

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Conflict of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Table and Figures

Table 1. PCL' MCs synthesis parameters: water and oil phases, temperature, stirring speed and synthesis duration.

MCs' acronym	Water phase (W ₁)* (wt.% of W ₁)	Oil phase (O)	Water phase (W ₂)	Temp. (°C)	Stirring speed (rpm)	Duration (hours)
M30(16)C(80)	5 ml of aqueous solution of Citric Acid (80wt.%)	PCL 30000 Mw 16wt.% in DCM			500	3
M45(16)C(80)	5 ml of aqueous solution of (80wt.%)	PCL 45000 Mw 16wt.% in DCM			500	3
M45(16)C(100)	5 gr of Citric Acid (100wt.%)	PCL 45000 Mw 16wt.% in DCM			600	1
M80(16)C(80)	5 ml of aqueous solution of (80wt.%)	PCL 80000 Mw 16wt.% in DCM	Water + PVA 2wt.% + GA 1.5wt.%	25 °C	500	1
M80(12)C(80)	5 ml of aqueous solution of (80wt.%)	PCL 80000 Mw 12wt.% in DCM			500	1
M30(16)N(5)	5 ml of aqueous solution of naringin (5wt.%)	PCL 30000 Mw 16wt.% in DCM			500	2
M45(16)N(1)	5 ml of aqueous solution of naringin (1wt.%)	PCL 45000 Mw 16wt.% in DCM			500	1
M45(16)N(5)	5 ml of aqueous solution of naringin (5wt.%)	PCL 45000 Mw 16wt.% in DCM			500	1.5
M80(16)N(5)	5 ml of aqueous solution of naringin (5wt.%)	PCL 80000 Mw 16wt.% in DCM			500	1
M80(12)N(5)	5 ml of aqueous solution of naringin (5wt.%)	PCL 80000 Mw 12wt.% in DCM			700	1

*Water phase 1 (W₁) consists of the compound to encapsulate dissolved in water. The W₁ was added to the O phase.

Table 2. PCL' MCs composition, encapsulation process yield (%) and mean size (μm), and respective standard deviation values, obtained from triplicates of each synthesis.

MCs' acronym	Final composition	Encapsulation process yield (%) \pm SD	Mean Size (μm) \pm SD
M30(16)C(80)	PCL (30000 Mw) 16% - Citric Acid (80%)	31.0 \pm 1.1	280 \pm 104
M45(16)C(80)	PCL (45000 Mw) 16% - Citric Acid (80%)	36.0 \pm 2.5	323 \pm 130
M45(16)C(100)	PCL (45000 Mw) 16% - Citric Acid (100%)	23.0 \pm 0.8	509 \pm 120
M80(16)C(80)	PCL (80000 Mw) 16% - Citric Acid (80%)	3.0 \pm 0.6	275 \pm 129
M80(12)C(80)	PCL (80000 Mw) 12% - Citric Acid (80%)	13.6 \pm 3.6	726 \pm 152
M30(16)N(5)	PCL (30000 Mw) 16% - Naringin (5%)	55.0 \pm 1.4	99 \pm 22
M45(16)N(1)	PCL (45000 Mw) 16% - Naringin (1%)	72.6 \pm 0.7	231 \pm 100
M45(16)N(5)	PCL (45000 Mw) 16% - Naringin (5%)	60.0 \pm 8.6	306 \pm 134
M80(16)N(5)	PCL (80000 Mw) 16% - Naringin (5%)	1.4 \pm 0.02	486 \pm 160
M80(12)N(5)	PCL (80000 Mw) 12% - Naringin (5%)	31.0 \pm 1.4	709 \pm 79

Table 3. PCL' MCs theoretical loading, experimental loading and encapsulation efficiency.

MCs acronym	Theoretical loading (%)	Experimental loading (%) from TGA	Encapsulation efficiency (%)
M30(16)C(80)	58.1	4	6.9
M45(16)C(80)	58.1	15	25.8
M45(16)C(100)	63.4	9	14.2
M80(12)C(80)	64.9	25	38.5
M30(16)N(5)	7.9	3	38.0
M45(16)N(5)	7.9	6	75.9
M80(12)N(5)	10.3	5	48.5

Table 4: Release kinetics parameters of different formulations at different pH solution and RE.

<i>Parameters</i>	<i>n</i>	<i>k</i>	<i>Correlation coefficient, r</i>
PCL30 (16)C(80)			
<i>pH 7</i>	0.59	0.56	0.99
<i>pH 5</i>	0.29	0.72	0.99
<i>pH 4</i>	0.37	0.7	0.99
<i>RE</i>	0.82	0.34	0.99
PCL45 (16)C(80)			
<i>pH 7</i>	0.13	0.7	0.92
<i>pH 5</i>	0.22	0.81	0.99
<i>pH 4</i>	0.3	0.88	0.97
<i>RE</i>	1.82	0.1	0.95
PCL80 (12)C(80)			
<i>pH 7</i>	0.02	0.96	0.92
<i>pH 5</i>	0.04	0.95	0.93
<i>pH 4</i>	0.03	0.93	0.97
<i>RE</i>	1.7	0.92	0.98
PCL30 (16)N(5)			
<i>pH 7</i>	0.23	0.91	0.97
<i>pH 5</i>	0.46	0.85	0.99
<i>pH 4</i>	0.7	0.88	0.99
<i>RE</i>	0.3	0.9	0.99
PCL45 (16)N(5)			
<i>pH 7</i>	0.5	0.6	0.97
<i>pH 5</i>	0.4	0.58	0.9
<i>pH 4</i>	0.45	0.5	0.99
<i>RE</i>	0.47	0.63	0.97
PCL80 (12)N(5)			
<i>pH 7</i>	1.84	0.08	0.94
<i>pH 5</i>	1.49	0.12	0.92
<i>pH 4</i>	1.54	0.13	0.95
<i>RE</i>	1.33	0.17	0.96

Fig. 1. SEM photomicrographs of the microcapsules. A (30x) and B (150x): M30(16)C(80); C (30x) and D (70x): M45(16)C(80); E (30x) and F (230x): M45(16)C(100); G (30x) and H (400x): M80(16)C(80); I (30x) and J (100x): M80(12)C(80); K (30x) and L (220x): M30(16)N(5); M (30x) and N (170x): M45(16)N(1); O (30x) and P (250x): M45(16)N(5); Q (30x) and R (190x): M80(16)N(5); S (25x) and T (130x): M80(12)N(5). The arrow in Fig. 1.P indicates the presence of naringin crystals encapsulated. The same happens for Fig. 1.L.

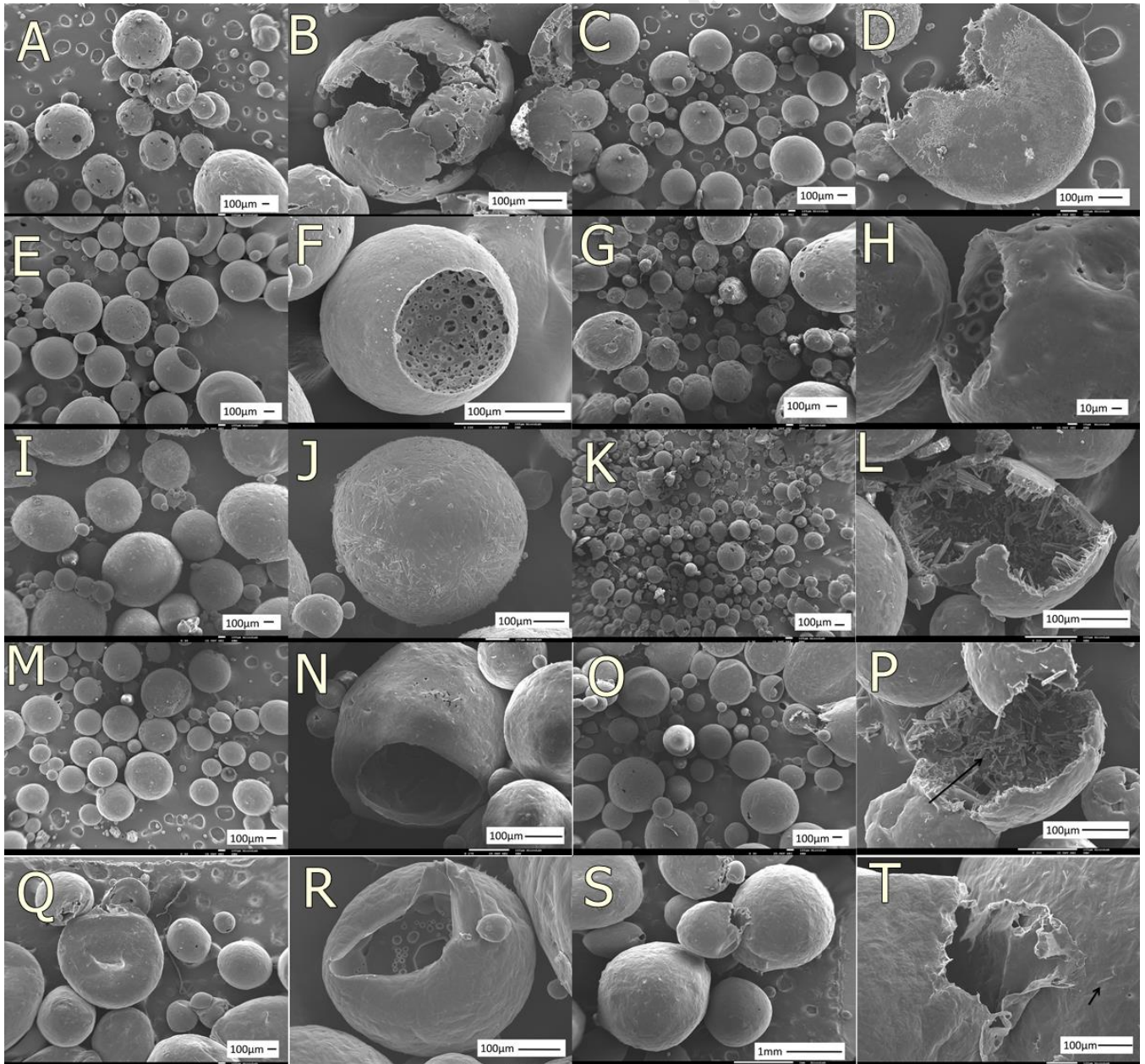


Fig. 2. Normalized FTIR-ATR spectra obtained from PCL 45000 Mw, encapsulated compound and resulting microcapsules. A: PCL MCs with citric acid; B: Magnification of the peaks observed in the region of interest, between 1850 and 1550 cm^{-1} ; C: PCL MCs with naringin; D: Magnification of the peaks observed in the region of interest, between 1850 and 1550 cm^{-1} .

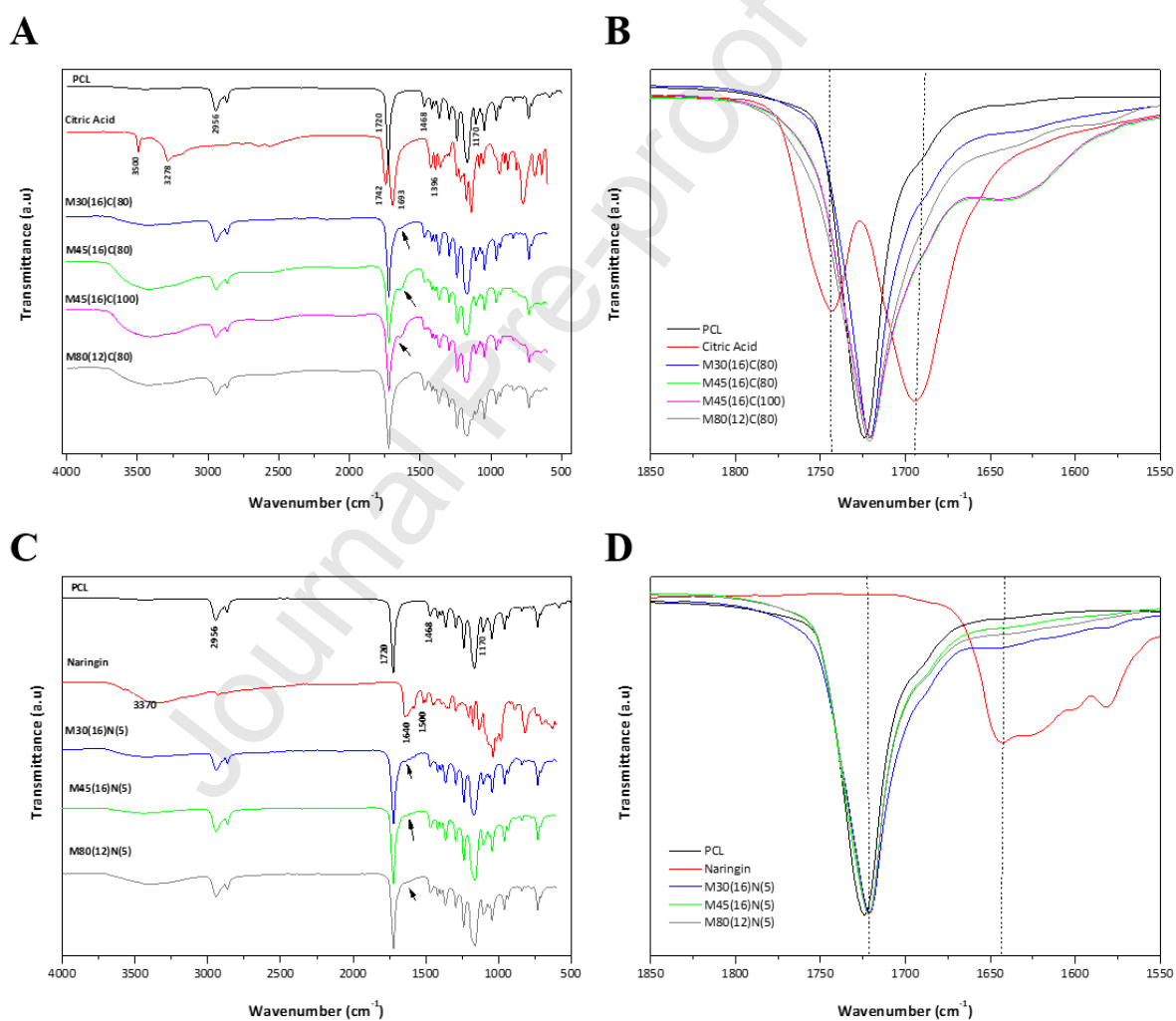


Fig. 3. TGA thermograms obtained from PCL 45000 Mw (shell material) and (A) citric acid and MCs prepared with citric acid and (B) naringin and MCs prepared with naringin.

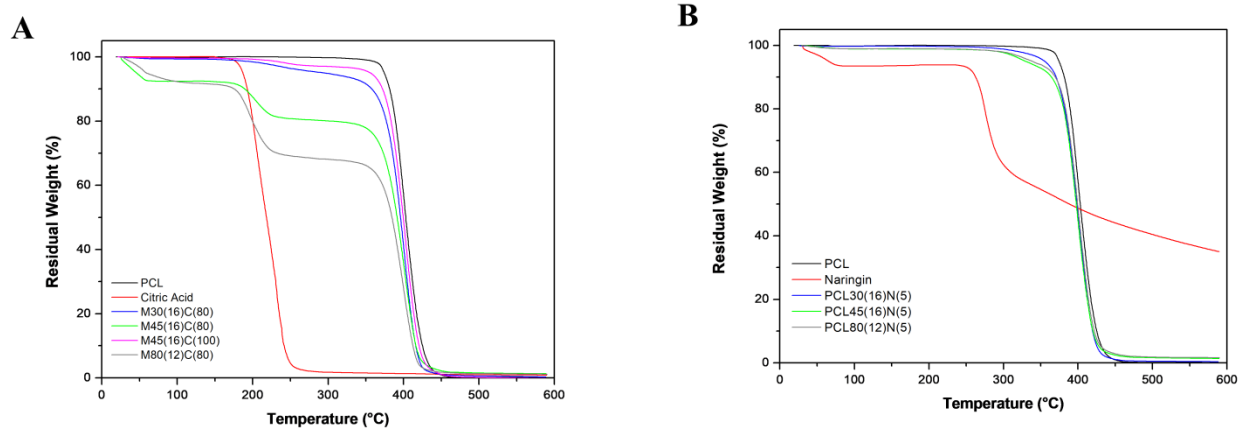
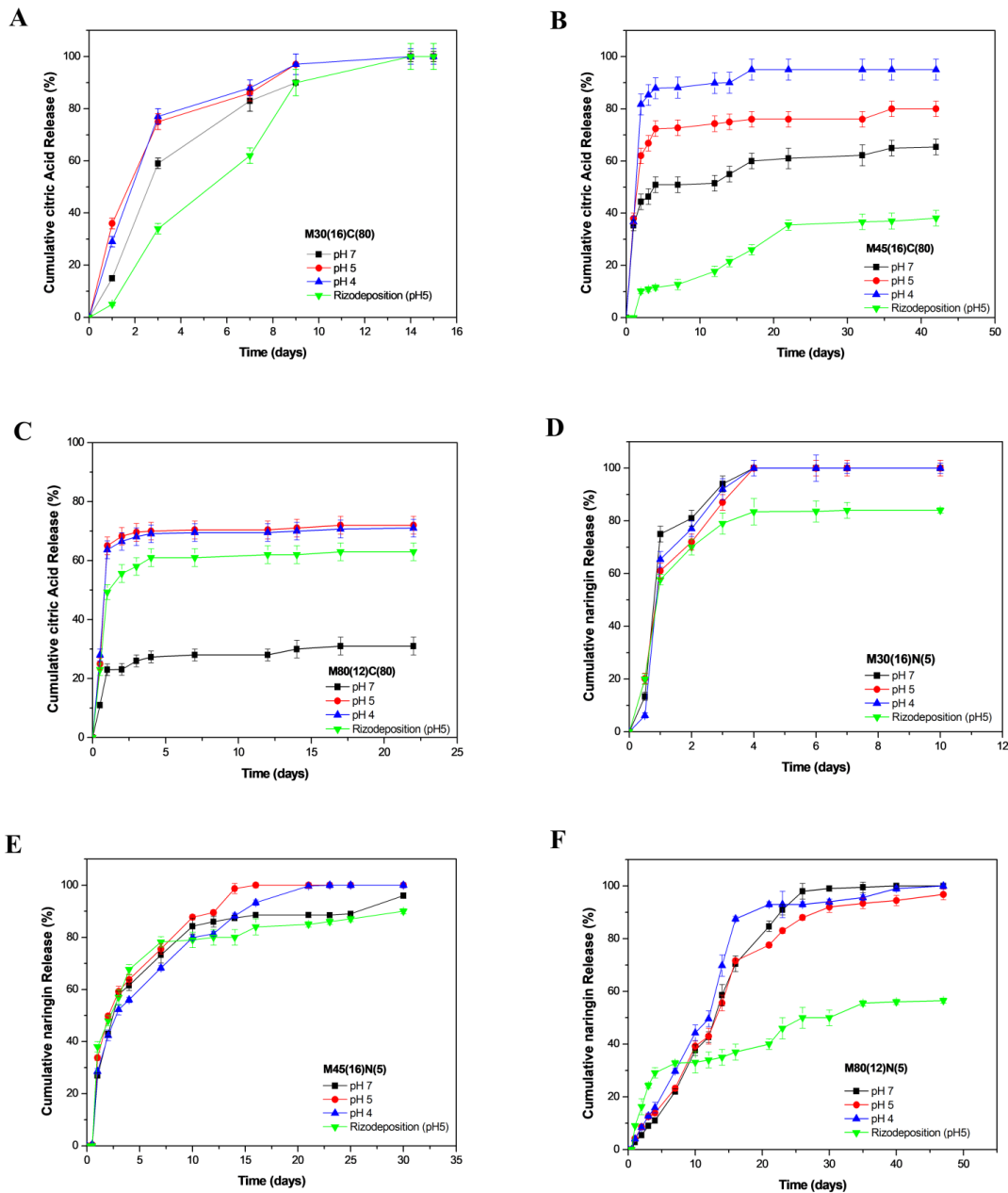


Fig. 4. Percentage of cumulative *in vitro* release profiles of (A) MCs containing citric acid: M30(16)C(80), M45(16)C(80) and M80(12)C(80); (B) MCs containing naringin: M30(16)N(5), M45(16)N(5) and M80(12)N(5), under different pH solutions. The release values displayed in the graphs are an average of three independent release experiments employing MCs of the same type, but from different batches (error bars represent standard deviations, n = 3).

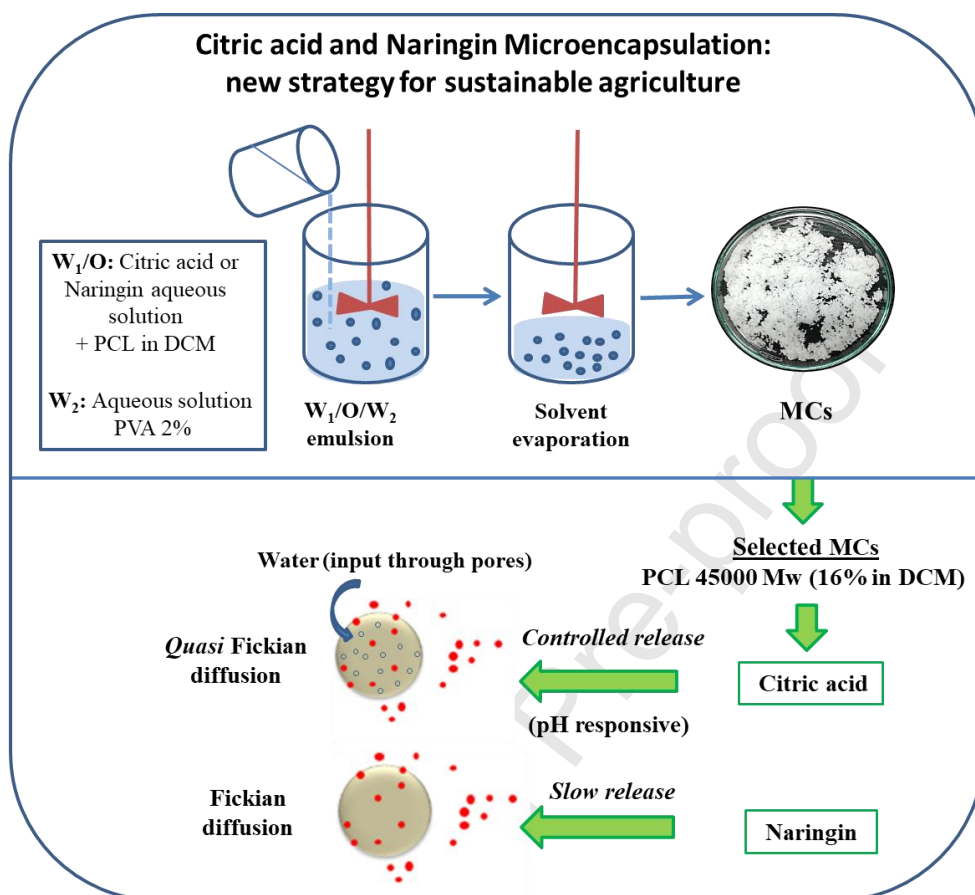


Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical Abstract



Highlights

- Successful encapsulation of citric acid and naringin with a biodegradable polymer PCL.
- Controlled release to improve the interaction between rhizobacteria and peanut plant.
- PCL Mw influences the release behavior.
- Slow release of naringin for 30 days, by diffusion, independent on pH of the medium.
- Controlled release of citric acid for 45 days (pH 4 > pH 5 > pH7 > RE).

Journal Pre-proof