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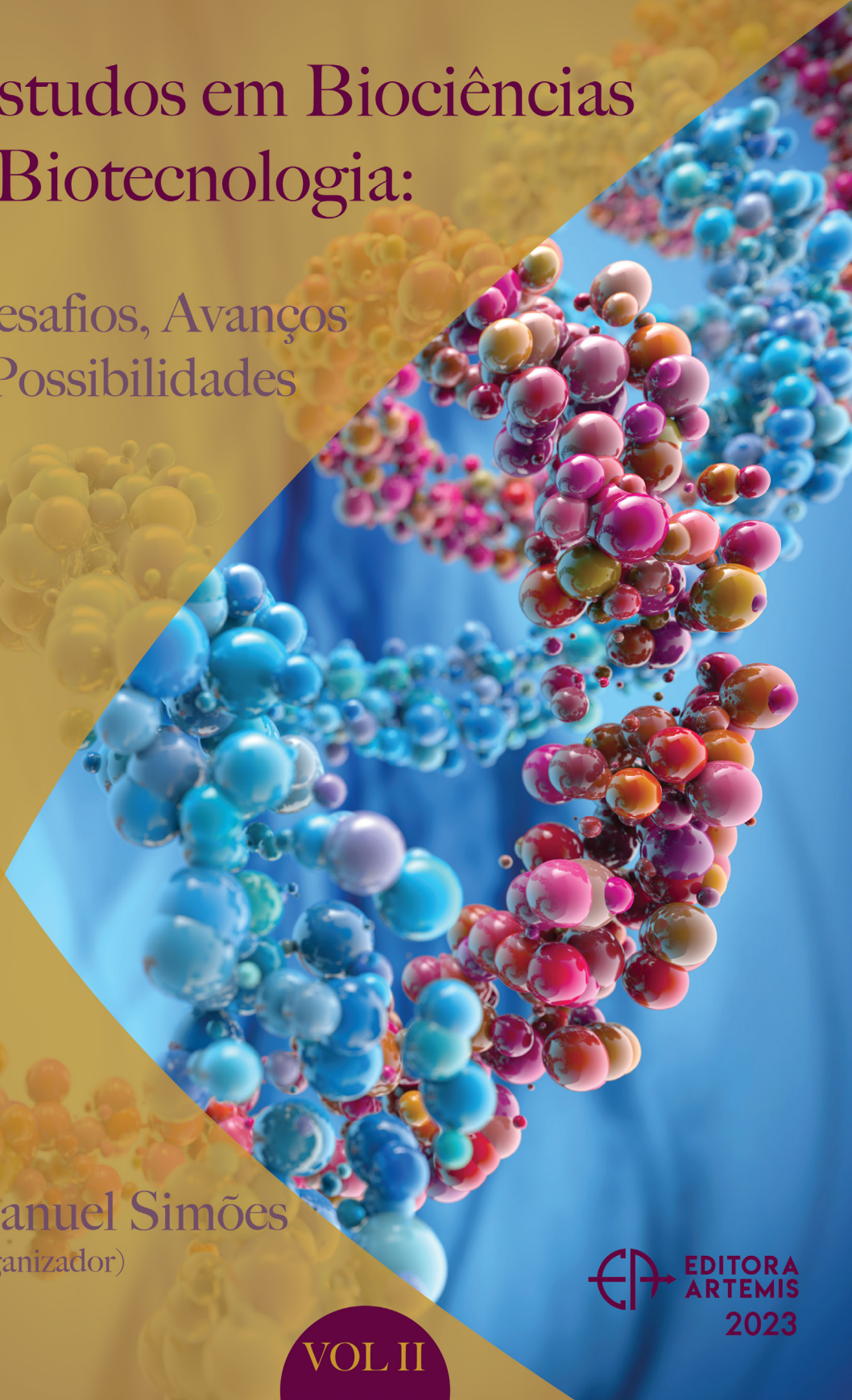
Desafios, Avanços e Possibilidades



Manuel Simões
(organizador)

EDITORA ARTEMIS
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VOL II

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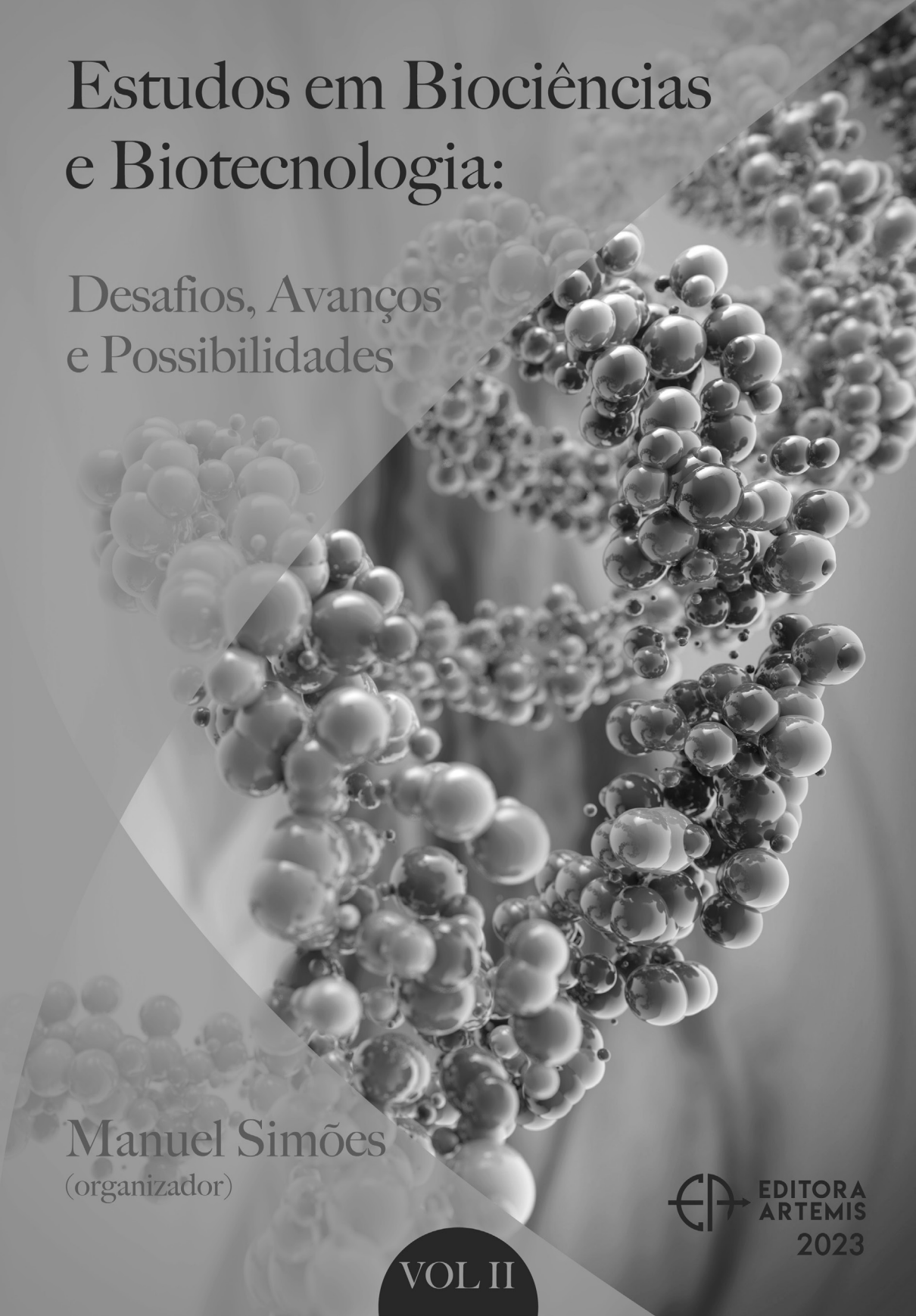

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PREFÁCIO

A investigação científica e o desenvolvimento tecnológico têm permitido criar soluções para os mais diversos problemas societais. Contudo, os avanços científicos e tecnológicos não se podem distanciar das abordagens de disseminação relevantes, que permitam que o conhecimento seja disponibilizado de forma criteriosa e compreensível à comunidade académica, às empresas/indústria e ao público em geral.

O segundo volume da edição "Estudos em Biociências e Biotecnologia" é composto por 12 capítulos que descrevem avanços significativos das ciências e tecnologias biológicas aplicadas a diversas áreas de investigação, complementando os trabalhos publicados no primeiro volume. Em particular, este volume, reúne capítulos relacionados com as ciências biológicas nas seguintes áreas/tópicos: biomédica (capítulos 1 e 2); biologia funcional e biotecnologia de plantas (capítulos 3 a 6); produção e proteção de alimentos (capítulos 7 a 9); ambiente e biorrecursos (capítulos 10 a 12).

O leitor deste volume beneficiará de um conjunto de informação inovadora que, além de ser um excelente contributo científico, contribuiu para dar resposta a diversos objetivos de desenvolvimento sustentável estabelecidos pela Assembleia Geral das Nações Unidas.

Manuel Simões

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ABSTRACT: A method for the extraction, purification, and quantification of gibberellic acid, GA3, from agricultural lands was develop using liquid chromatography HPLC. Soil samples, taken at 15 and 30 cm deep, from farmlands with wheat, vegetable, and alfalfa crops were analyzed to quantify GA3 in the range of between 39 to 100 mg/ kg of dry soil. The presence of GA3 was monitored in a commercial white chickpea field. The presence of the phytohormone is affected by farming activities, as GA3 could still be detected on the soil, but it was mainly found in the roots, reaching up to 15550 mg/kg of dry root. The interactions between crops and microorganisms present in the soil and roots can be studied via this analytical method.
KEYWORDS: Gibberellic acid. HPLC. Agricultural soil.

1 INTRODUCTION

The gibberellins are phytohormones that foster the growth and development of plants and their fruits. The gibberellic acid GA3 is the most abundant and commercially relevant of the gibberellins (Idbal et al. 2001). The GA3 is synthesized by filamentous fungi and phytopathogens as *Gibberella fujikuroi*. It is also found in vegetable tissue and exudates, but the specific site where plants synthesize it has not been determined (Grupta &Chakrabarty, 2013). The use of GA3 in farming is limited due to its high price, despite its benefits. Alternative production methods, such as solid state fermentation of agri-food waste as a substrate, are studied to find an affordable option (Rodrigues et al. 2011).

The GA3 has been used in different crops via foliar spray and dipping treatment; in potato crops it is used to reduce the dormancy periods and foster the growth of shoots, using GA3 at 750 to 1000 ppm and 40 to 50 ppm, respectively (Kabede-Asalfew, 2016). In blackberry crops, a foliar spray using GA3 at 5 and 10 ppm, resulted in a significant increment in the number, weight and size of the fruit; in chickpea crops, GA3 was applied at concentrations of 10 and 20 mg/L. It was found that applying it at the start of the plant flowering enhances the development of foliage and flowers (Çolak, 2018). Gavino et al. (2008) pointed out that in China, rice crops use GA3 at a dose of 150 to 300 g ha^{-1}, while in other countries only 45 – 50 g ha^{-1} are used due to the high cost of the phytohormone.

Several techniques have been developed for the extraction, purification, and quantification of this phytohormone due to its importance. David et al. (2017) extracted and quantified GA3 from maize kernels, using different solutions of acetonitrile and methanol in water acidified with formic acid at 5%, and pure ethyl acetate as solvents for the extraction. They used a solvent-sample ratio of 20mL by 10 g, respectively. The lixiviation process was done by means of an orbital shaker and ultrasonification; obtaining analite concentrations of 15.06 and 30.01 mg/ kg of sample. The acetonitrile – formic acid at 5% (80:20) was the best solvent for the extraction. Manzi et al. (2015)

extracted and quantified gibberellins from tangerine fruitlets, dehydrated by immersion in liquid nitrogen. A powdered sample of 200 mg was mixed with a solution of isopropanol – acetic acid (99:1), the extract was analized via liquid chromatography UPLC MS/MS, identifying residues of GA1, GA3, GA4, and GA7 in concentrations of between 0.7 – 56.5 ng/g of dry sample. New analytical techniques allow for the detection and quantification of these hormones in several plants and their tissues. These analytical techniques must help comprehend the synthesis and the role of these compounds in the plants (Garmendia et al. 2019).

The objective of this work is an extraction and quantification technique to assess GA3 in agricultural soils, that could foster the understanding of the different biological phenomena that take place in agricultural soils because of the microorganisms-plant interactions on the surface of the roots. The technique was used on soils from farmlands with wheat, vegetables, alfalfa, and white chickpea crops. In this last crop the concentration of GA3 was monitored through the crop cycle.

2. METHODS

2.1 SOILS

The soils samples were collected from a farm belonging to Mexico's Institute of Technology Roque (Tecnológico Nacional de Mexico in Roque) located in Celaya, Gto. (20°34'53.15"N, 100°49'36.47"W), and a farm in Santo Tomas (Salvatierra, Gto. 20° 15'07.48"N, 100°55'29.49"W). The identifiers for all the simples considered in this study are presented in Table 1. The sampling technique considered 5 sampling points per hectare at depths of 15 and 30 cm (Espinosa et al. 2012). The samples taken from 5 points, approximately 2 kg, were mixed and dried at room temperature in a drying chamber equipped with an air blower. Out of the 2kg sample, one kg was selected and stored at 10°C for further analysis. Additionally, a sample of compost, bought in the city of Celaya, was also analyzed.

Table 1. Description, source, and sample identifier.

ID	Crop	Depth(cm)	Source
SA15	Alfalfa	15	Roque
SA30	Alfalfa	30	Roque
SH15	Vegetables	15	Roque
SH30	Vegetables	30	Roque
ST15	Wheat	15	Roque
ST30	Wheat	30	Roque

ID	Crop	Depth(cm)	Source
SG15	Chickpea	15	Santo Tomas
SG30	Chickpea	30	Santo Tomas
SC	Compost		Celaya

The soils underwent a physicochemical characterization according to the following parameters: texture (USDA-2014), water retention: saturation percentage (%S) and soil capacity (CC), apparent density, true density, pH, and organic matter content (NOM -021-RECNAT-2000).

2.2 EXTRACTION OF GIBBERELLIC ACID FROM THE SOIL

The dry soil samples were ground in a ball mill and separated using a sieve equipped with a 100 mesh. A 65 and 35 % (v/v) solution of methanol and water acidified with 1% formic acid was used as solvent. The extraction was carried out in a sample:solvent ratio of 5g:30 mL. The solvents used are HPLC grade (Karal ®). The sample: solvent mixture was stirred for 10 minutes using a magnetic mixer at 200 rpm, after that the mixture was subjected to sonification for 10 minutes (Branson 1800). The resulting suspension remained undisturbed for 24 h at 10°C. The supernatant was decanted and centrifuged at 6500 rpm for 5 minutes (Ohaus FC57706W). A 10 mL aliquot was filtered using a 0.45µm cellulose acetate membrane and stored in an amber vial at 10°C for its posterior HPLC analysis.

2.3 CHROMATOGRAPHIC METHOD

A liquid chromatograph HPLC Agilent 1200 ® equipped with a diode array detector (DAD) was used. A Discovery ® HS-C18 Sigma-Aldrich 5 µm 4.6 mm x 25 cm was utilized. As the mobile phase a methanol – water acidified with formic acid at 1% solution in a 35:65 ratio was employed (Karal ®). A constant flow of 1 mL/minute of the mobile phase was fed to the column; 50 µL were injected and a detection length wave of 245 nm was utilized.

A stock standard solution made with Gibberellin A3 (48880-5G-F Sigma Science®) dissolved in the mobile phase, in a 1000 mg/L concentration, was prepared. From this stock solution, standards with concentration ranging from 1 to 10 mg/L and 50 to 500 mg/L were prepared, and used to obtain calibration curves. The instrumental detection and quantification limits were determined (Quino et al. 2007). The method accuracy was blind tested by two independent analysts that were provided with a sample of a standard solution. Each analyst reported 5 concentration estimations to

the sample solution. The method accuracy is accepted if the percentage of recovery is of between 90 to 110 % of recovery. The procedure based on the Horwitsz equation and relation was used to estimate the repeatability and reproducibility of the method, and the acceptance or rejection criteria of the analytical method variability (Rivera-Orozco & Rodríguez-Baéz, 2010).

The concentration of GA3 in soils was evaluated by analyzing soil samples before and after being fortified with GA3, this provided an estimate of the percentage of recovery for the extraction and extract purification processes.

2.4 ANALYSIS OF GIBBERELLINS IN SOILS DURING CHICKPEA FARMING

The usefullness of the technique developed was demonstrated by testing and quantifying GA3 in a conventional chickpea crop cycle from January to April, 2019 in a farm located in Santo Tomas in the municipality of Salvatierra, Gto. The technique was also used to assess gibberellins in the chickpea roots and foliage.

3 RESULTS AND DISCUSSION

Figure 1 shows the calibration curve for GA3, and Figure 2 presents a chromatogram for a GA3 standard where its chromatographic peak is observed with a retention time of 3.987 ±0 .25 minutes, the spectrum for UV absorption is also shown with a peak of maximum absorption at 254 nm. In the accuracy test, analyst 1 obtained a recovery percentage of 96.61% with a standard deviation relative to the mean or variation coefficient of 0.98%, and absolute error of 3.38%; while analyst 2 obtained a recovery percentage of 97.08% with a variation coefficient and absolute error of 1.15% and 2.91%, respectively. Since the variation coefficients are less than 3.45%, as obtained with the Horwitz equation, the chromatographic method can be deemed as accurate. Horwitz equation is an empirical expression that correlates the expected variation coefficients when analyte samples at a specific concentration are analyzed. The data for the creation and validation of this equation was gathered from analytical test results from well-known laboratories (Rivera-Orozco y Rodríguez-Baéz,2010). Regarding the repeatability of the method, analysts 1 and 2 obtained Horwitz ratios (HorRat, variation coefficient ratio: exterimental divided by the Horwitz variation coefficient) of 0.2850 and 0.3341, respectively. Since the Horwitz ratios are in the range 0.3< HorRat < 1.3, the chromatographic technique is considered repeatable and reproducible. And for the reproducibility between analysts, a variation coefficient of 0.7330% was obtained, with a HorRat coefficient of 0.3090. Thus, the method is considered reproducible.

Figure 1. Calibration curve of the GA3 standard in a methanol- water acidified with formic acid 1%, in a 35:65 ratio, at 254 nm detection lengthwave.

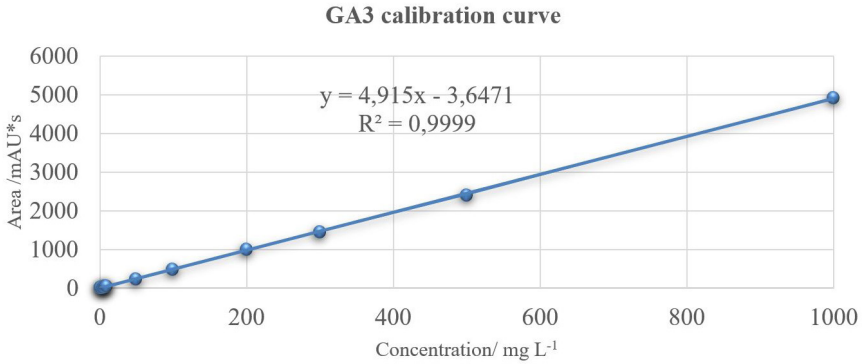
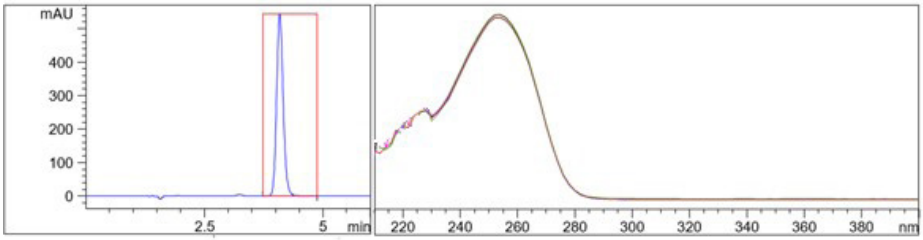


Figure 2. Chromatogram of GA3 standard at 254nm y characteristic UV absortion spectrum.



The characteristics of the soils considered in this study are presented in Table 2. Roque's soil is classified as a loamy sand soil, while Santo Tomas is a loam soil, and the compost is considered sandy. The loam and loamy sand soils have an optimal texture for agriculture (FAO-2009). The soils in Santo Tomas possesses the best characteristics for agriculture, since it contains enough organic matter, an almost neutral pH, and a high water-retention capacity. The compost contained large particles vegetable particles, but a extremely low water-retention capacity.

Table 2. Physico-chemical characterization of the selected soils.

Sample origin	Particle size (% p/p)			Apparent density (g/mL)	True density (g/mL)	pH	Organic matter (%)	Water retention (g of water/100 g of soil)	
	Clay	Silt	Sand					CC	% S
Roque, Celaya.	34.71	2.70	62.58	1.1431	2.2228	8.11	4.52	38.39	67.19
Santo Tomas, Salvatierra.	43.08	1.12	55.80	1.8186	2.8577	7.56	7.52	46.77	81.85
Compost.	11.984	1.136	86.88	0.6073	0.3232	7.63	60.65	10.37	18.15

The diazothrophic bacteria in the soil around the plant root system are characterized for binding nitrogen to the soil, and to produce phytohormones. The bacteria of the genera *Azotobacter* and *Azospirillum* are capable of producing several hormones, such as, indoleacetic acid, gibberellic acid, abscisic acid, and ethylene (Narula et al. 2006). Due to the nature of these microorganisms to bind nitrogen and produce phytohormones, fostering plant development and increasing the yield in crops such as maize and wheat, several commercial inoculants were developed that can be applied to the roots of these crops (Arshad y Frankenberger, 1991). The concentration of GA3 in the soils of the crops considered, namely wheat, vegetables (broccoli), and alfalfa, are presented in Table 3. No GA3 was detected in the compost. According to Sundberg and Jönsson (2008), the pH during compost production is too low due to the high organic matter content, which inhibits bacterial activity. Additionally, compost is made from vegetable waste, while bacteria in the soil have a symbiotic relationship the plant roots.

The soil sample from the wheat farm was taker just after the wheat was threshed, when the wheat roots were still in the soil. In the vegetable farmland, the soil was sampled after fallowing and plowing the land once the broccoli was harvested. In this case, the plant roots were mechanically removed and destroyed. As for the alfalfa, since this is a crop that regrows after being harvested for the first time, the root system was intact. Despite the fact that the plants are capable of synthetizing gibberellins, the precise bioactive site has not been identified, as neither if the hormones travel inside the plant to trigger plant growth, flowering, and shoots formation (Gupta y Chakrabarty, 2013). The presence of gibberellins in the soil around the roots come mainly from microorganisms, that have developed a symbiotic relationship with the plants.

Table 3. Gibberellic acid concentration in agricultural soils.

Soil sample	Concentration mg kg^{-1}
ST15	83.9
ST30	76.17
SH15	39.4
SH30	49.5
SA15	102.1
SA30	63.4
SC	Not Detected

3.1 GA3 DURING CHICKPEA CROP CYCLE

Samples of chickpeas, of the Sinaloa variety, and its agricultural soil were taken during the crop cycle, the sample was obtained from the center of one hectare, sampling 5 plants and its soil at 15 and 30 cm deep. The roots and foliage were dried

at room temperature with a continuous air flow. The vegetable matrices were subjected to the same extraction procedure as the soils. Table 4 shows the different agricultural activities performed, and the GA3 concentration found in the samples analyzed. The GA3 concentration was low in general, with the exception of the sample at 30 cm deep, taken just after the seeding, plant germination and emergence. No GA3 was found in the foliage, the roots did showed variable concentrations of this phytohormone, with a maximum just after an emergency surface irrigation of the crop, and a foliar spray of phytohormones and pesticides to the plants. Normally, the chickpea field is not irrigated after the seeding, unless it is subject to heat and hydric stress. Then an emergency surface irrigation takes place, at the risk of suffocating the roots and killing the plants (Morard et al. 2000). In the crop considered in this study, about 20% of the plants died due to this. Samples taken from the roots of the dead plants presented a dark color and no GA3 could be detected. The chickpea was harvested while still fresh in the pods, the healthy plants presented pale roots, its central body had a length of between 25 to 30 cm, and abundant radial nodules.

Table 4. Agricultural activities during chickpea crop cycle and GA3 determination (mg/kg dry basis).

Date	Agricultural activity	Soil 15 cm	Soil 30cm	Root	Foliage
16 /11/2018	Maize harvest				
	Sampling	17	20	NM	NM
6/01/2019	Mechanical plowing of the soil				
10/01/2019	Surface irrigation				
31/01/2019	Mechanical seeding 120 kg/ha				
14/02/2019	Sampling, 5-7 cm plants	17	1700	700	ND
24/02/2019	Sampling, 10-15cm plants	53	30	1050	ND
02/03/2019	Fumigation Dimethoate 1L/ha Enzymatic extract: 1L/ha gibberellins 32.2 ppm; indoleacetic acid 32.2 ppm; zeatin 83.2 ppm.				
16/03/2019	Sampling, 30-32cm plants	18	10	50	ND
18/03/2019	Surface irrigation				
1 /04 /2019	Fumigation Dimethoate 1L/ha Solution with gibberellic acid 4% and micronutrients 1L/ha				
5/04/2019	Sampling, 40-42 cm plants with incipient flowers and pods	35	10	1550	ND
25/04/2019	Sampling, 37-42cm plants with fully developed pods.			850	ND
26-30/04/2019	Harvest of fresh chickpea pods				

4 CONCLUSIONS

A simple and reliable analytical method for the extraction, purification and quantification of gibberellic acid in agricultural soils was developed. The method was tested in different soils with various crops, finding that the procedure allows the quantification of GA2 in concentrations of between 17 to 1700 mg/ kg of dry soil. The presence of GA3 is an indicator of the effect that a agricultural activities have on the root-soil interactions.

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SOBRE O ORGANIZADOR

Manuel Simões é licenciado em Engenharia Biológica e doutorado em Engenharia Química e Biológica. Atualmente é Professor Associado com Agregação e Pró-Diretor da Faculdade de Engenharia da Universidade do Porto (FEUP), e investigador sénior do Laboratório de Engenharia de Processos, Ambiente, Biotecnologia e Energia (LEPABE) do Departamento de Engenharia Química da FEUP. Nos últimos anos esteve envolvido em 10 projetos nacionais (5 como investigador principal) e 6 projetos europeus. Foi membro do comité de gestão da ação COST BACFOODNET (Rede Europeia para Mitigação da Colonização e Persistência Bacteriana em Alimentos e Ambientes de Processamento de Alimentos) e esteve envolvido em outras 2 ações: iPROMEDAI e MUTALIG. Manuel Simões tem mais de 190 artigos publicados em revistas indexadas no Journal of Citation Reports, 4 livros (1 como autor e 3 como editor) e mais de 40 capítulos em livros. Ele é Editor Associado para o jornal Biofouling - The Journal of Bioadhesion and Biofilm Research (o periódico mais antigo sobre pesquisa em biofilme), Editor Associado para o jornal Frontiers in Microbiology e Section Editor-in-Chief para o jornal Antibiotics. Seus principais interesses de pesquisa estão focados nos mecanismos de formação de biofilme e seu controlo com agentes antimicrobianos, particularmente usando novas moléculas antimicrobianas, e no uso de microalgas para tratamento de efluentes. É um dos investigadores mais citados do mundo (top 1%), tendo sido distinguido nos últimos dois anos no índice Essential Science Indicators, um dos mais prestigiados indicadores da qualidade de investigação.

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