

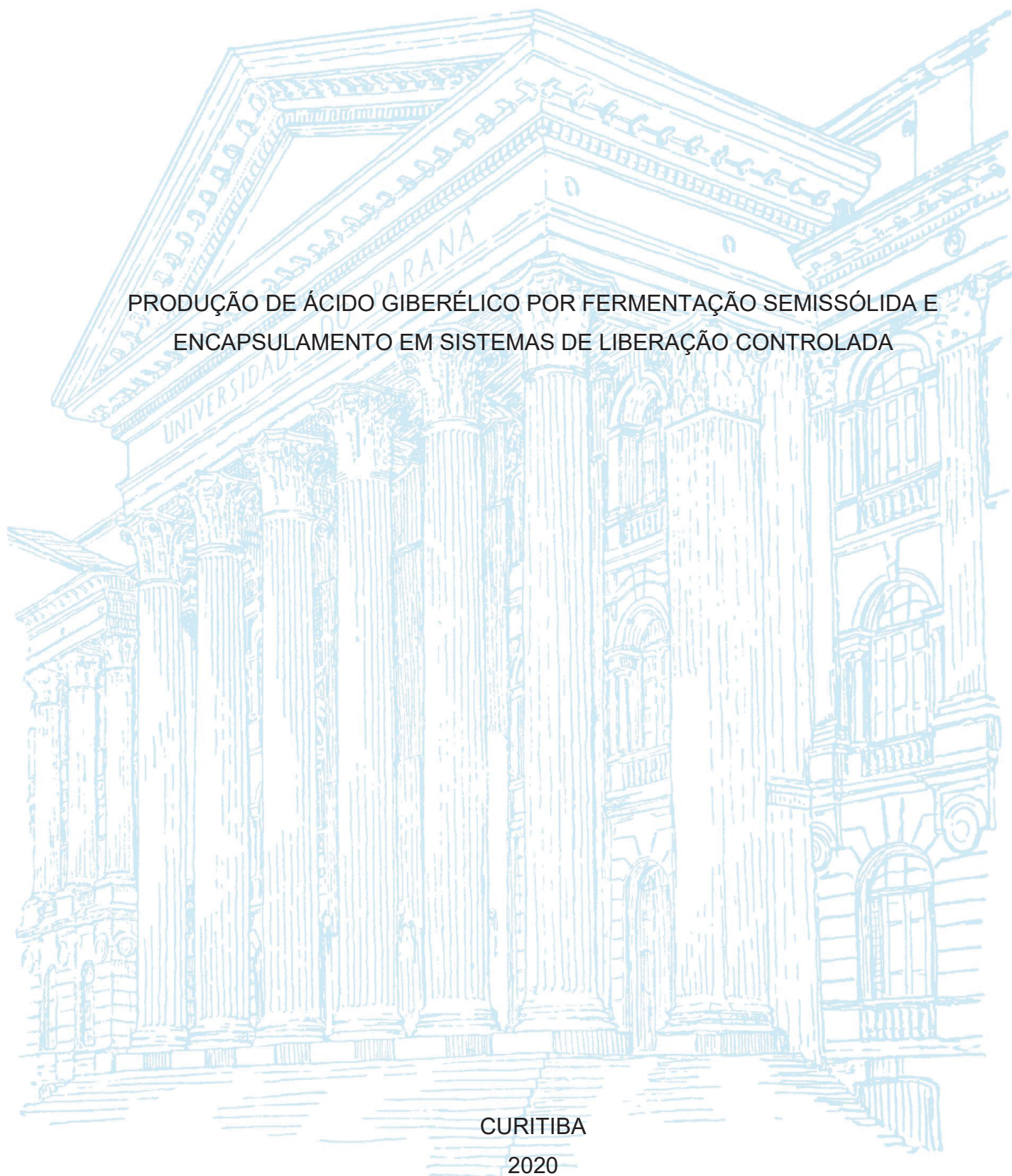
UNIVERSIDADE FEDERAL DO PARANÁ

GABRIELLE CAMILLE SEXTOS

PRODUÇÃO DE ÁCIDO GIBERÉLICO POR FERMENTAÇÃO SEMISSÓLIDA E
ENCAPSULAMENTO EM SISTEMAS DE LIBERAÇÃO CONTROLADA

CURITIBA

2020



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ENCAPSULAMENTO EM SISTEMAS DE LIBERAÇÃO CONTROLADA

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Orientadora: Profa. Dra. Luciana Porto de Souza Vandenberghe

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Dedico aos meus pais, Kellen e Emerson, à
minha irmã, Polliana, e ao meu namorado,
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*“Education is the most powerful weapon
which you can use to change the world.”*

(Nelson Mandela)

RESUMO

O ácido giberélico (GA₃) é um hormônio vegetal bastante conhecido do grupo das giberelinas, sendo responsável pelo desenvolvimento e crescimento das plantas. O presente trabalho descreve novos avanços na produção biotecnológica, purificação e formulação do GA₃. Com destaque para a síntese de nanopartículas, que possibilitam a liberação controlada da biomolécula, melhorando a sua estabilidade e atividade. Além disso, experimentos foram conduzidos, o GA₃ foi produzido por meio de fermentação semissólida do fungo *Gibberella fujikuroi* utilizando a polpa cítrica e a casca de soja como substrato. Um estudo para determinar a quantidade de sacarose e ureia adicionados ao meio foi realizado. A maior produção de GA₃ em frascos foi de 154,44 mg/L com um meio de composição de 3,5%(m/v) de polpa cítrica, 1,5% (m/v) de casca de soja, 180 g/L de sacarose e 4 g/L de ureia. Com o intuito de estudar o escalonamento do processo, fermentações em biorreatores do tipo coluna de bolhas e STR foram realizadas, as concentrações de GA₃ obtidas foram de 183,31 mg/L e 217,67 mg/L, respectivamente. O GA₃ purificado foi encapsulado em diferentes sistemas. Os biopolímeros testados foram a quitosana, alginato e kefiran, em conjunto ou não, para a formação de beads e nanopartículas. Os sistemas, com e sem GA₃, foram testados em cultivar de soja, quanto ao impacto na germinação das sementes e no comprimento das raízes. Beads de alginato com GA₃ e alginato-kefiran com GA₃, e micropartículas de quitosana com GA₃ foram obtidas. Todas as sementes germinaram após serem expostas aos beads de alginato com GA₃, sendo observado com o controle uma porcentagem de germinação de apenas 40%. As sementes que foram colocadas junto aos beads de alginato-kefiran com GA₃ apresentaram o maior comprimento de raízes. A influência positiva obtida pela aplicação da biomolécula nos cultivos gera um ganho de produtividade que impacta economicamente muitos países com base na agroindústria.

Palavras-chave: Ácido giberélico. Fermentação semissólida. Formulação. Encapsulamento. Liberação Controlada.

ABSTRACT

Gibberellic acid (GA₃) is a plant hormone well known from gibberellins group that is responsible for the development and growth of plants. The present study describes the new advances in biotechnological production, purification, and formulation of GA₃. Focus on nanoparticle synthesis that allows the controlled delivery of the biomolecule improving its stability and activity. Besides that, experiments were carried out; the GA₃ was produced by semisolid fermentation of the fungi *Gibberella fujikuroi* using citric pulp and soybean husk as substrate. A study to determine the quantity of sucrose and urea added to the medium was done. The highest GA₃ production in flasks was 154.44 mg/L using a medium composition of 3.5 % (w/v) of citric pulp, 1.5% (w/v) of soybean husk, 180 g/L of sucrose and 4 g/L of urea. In order to study the scale-up of the process, fermentations were conducted in bubble column bioreactor and STR, the GA₃ concentrations obtained were 183.31 mg/L and 217.67 mg/L, respectively. The purified GA₃ was encapsulated in different systems. The biopolymers tested were chitosan, alginate and kefiran, combined or not, to the formation of beads and nanoparticles. The systems, with and without GA₃, were tested in soybean cultivar for the impact in seeds germinations and in root length. Beads of alginate with GA₃ and alginate-kefiran with GA₃, and microparticles of chitosan with GA₃ were produced. All seeds germinated after being exposed to the alginate with GA₃ beads, while the control showed a germination percentage of only 40%. The seeds that were placed with the alginate-kefiran with GA₃ beads presented the highest root length. The positive influence observed by the biomolecule application in cultivars results in gain of productivity that economically impact countries based on the agro-industry.

Keywords: Gibberellic acid. Semisolid fermentation. Formulation. Encapsulation. Controlled delivery.

FIGURE INDEX

FIGURE 1.	Steps of GA ₃ industrial production and formulation.....	18
FIGURE 2.	Diagram of nanostructures for bioactive molecule delivery systems.....	28
FIGURE 3.	Methods of encapsulation based on chitosan used as the nanomaterial: I) Emulsion cross-linking; II) Emulsion-droplet coalescence; III) Ionotropic gelation; IV) Precipitation; V) Reverse micelles; VI) Sieving; VII) Spray drying. NPs: nanoparticles.....	30
FIGURE 4.	The ionotropic pregelation and gelation methods for GA ₃ encapsulation. CaCl ₂ : calcium chloride; GA ₃ : gibberellic acid; ALG/CS: alginate and chitosan; TPP: tripolyphosphate; CS/TPP: chitosan and tripolyphosphate.....	32
FIGURE 5.	The covalent attachment technique for GA ₃ -CS-nanoparticles formation. GA ₃ : gibberellic acid; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; NHS: N-hydroxysuccinimide; CS: chitosan.....	34
FIGURE 6.	GA ₃ production, encapsulation and application steps.....	41
FIGURE 7.	GA ₃ production by SSSF using CP/SH medium with the addition of different concentrations of sucrose and urea.....	49
FIGURE 8.	Kinetics of GA ₃ production with different aeration rates in BCR.....	49
FIGURE 9.	Kinetics of GA ₃ production and sugar consumption in STR using CP/SH medium.....	51
FIGURE 10.	SEM images of beads: ALG at 250 x (A) and 2 kx (B); ALG-GA ₃ at 250 x (C) and 2 kx (D); ALG/KEF at 250 x (E) and 2 kx (F); and ALG/KEF-GA ₃ at 250 x (G) and 2 kx (H).....	52
FIGURE 11.	SEM images of nanoparticles: CS at 500 x (A) and 5 kx (B); CS-GA ₃ at 500 x (C) and 5 kx (D); CS/KEF at 500 x (E) and 5 kx (F); and CS/KEF- GA ₃ at 500 x (G) and 5 kx (H).....	53
FIGURE 12.	Release profiles of GA ₃ encapsulated in ALG (A) and ALG/KEF (B) beads.....	54

TABLE INDEX

TABLE 1. Recent GA ₃ production using different alternative substrate and fermentation techniques.	20
TABLE 2. GA ₃ commercial products available in the market.	22
TABLE 3. Different additives used in GA ₃ liquid formulation.	25
TABLE 4. Different types of ingredients for solid formulation and examples of chemicals.	27
TABLE 5. Matrices employed as GA ₃ delivery system and their characteristics.	35
TABLE 6. The germination percentage, mean germination time, and root length of soybean for each treatment for the first germination assay.	56
TABLE 7. The germination percentage, mean germination time, and root length of soybean for each treatment for the second germination assay.	57

ABBREVIATIONS AND ACRONYMS INDEX

ALG	– Alginate;
ANOVA	– Analysis of Variance;
BCR	– Bubble Column Reactor;
CaCl ₂	– Calcium chloride;
CCRD	– Central Composite Rotational Design;
CS	– Chitosan;
CP	– Citric pulp;
CP/SHAE	– Citric Pulp and Soybean Husks Aqueous Extract;
GA	– Gibberellin;
GA ₃	– Gibberellic acid;
HCl	– Hydrochloric acid;
HPLC	– High Performance Liquid Chromatography;
IBGE	– <i>Instituto Brasileiro de Geografia e Estatística</i> ;
KCl	– Potassium chloride;
KEF	– Kefiran;
PDA	– Potato Dextrose Agar;
PGR	– Plant Growth Regulator;
SEM	– Scanning electron microscopy;
SH	– Soybean husk;
SHAE	– Soybean Husks Aqueous Extract;
SmF	– Submerged Fermentation;
SSF	– Solid-State Fermentation;
SSSF	– Semi-Solid State Fermentation;
STR	– Stirred Tank Reactor;
TPP	– Tripolyphosphate;
VOC	– Volatile Organic Compounds;
w/v	– weight/volume;
γ-PGA	– poly-γ-glutamic acid.

SUMMARY

1	INTRODUCTION	14
2	CHAPTER 1: STRATEGIES FOR PLANT GROWTH HORMONE'S (GIBBERELIC ACID) STABILITY - PRODUCTION, FORMULATION AND ENCAPSULATION USING NANOPARTICLES	16
2.1	ABSTRACT	16
2.2	INTRODUCTION	16
2.3	GA ₃ PRODUCTION BY FERMENTATION	18
2.4	GA ₃ SEPARATION AND PURIFICATION PROCESSES	20
2.5	GA ₃ PRODUCT FORMULATION	21
2.5.1	GA ₃ liquid formulation	22
2.5.2	GA ₃ solid formulation	24
2.6	ENCAPSULATION METHODS FOR GA ₃ CONTROLLED RELEASE	27
2.7	CONCLUSIONS	36
3	CHAPTER 2. STUDY OF DIFFERENT SYSTEMS FOR CONTROLLED DELIVERY OF GIBBERELIC ACID PRODUCED IN SUBMERGED FERMENTATION BY <i>GIBBERELLA FUJIKUROI</i>.....	37
3.1	ABSTRACT	37
3.2	INTRODUCTION	37
3.3	OBJECTIVES	40
3.3.1	Specific objectives	41
3.4	MATERIAL AND METHODS	41
3.4.1	Microorganism	41
3.4.2	Substrates	41
3.4.3	Inoculum preparation	42
3.4.4	Optimization of inoculum production.....	42
3.4.5	Optimization of GA ₃ production by semisolid fermentation (SSSF)	42
3.4.6	GA ₃ production by SSSF in bubble column reactor (BCR).....	42
3.4.7	GA ₃ production by SSSF in stirred tank reactor (STR)	43
3.4.8	Analysis of GA ₃ production	43
3.4.9	Analysis of reducing sugars	43
3.4.10	Membrane Separation and purification of GA ₃	43
3.4.11	Kefiran (KEF) production and extraction.....	44

3.4.12	Preparation of GA ₃ alginate beads (ALG).....	44
3.4.13	Preparation of GA ₃ alginate-kefiran beads (ALG/KEF)	44
3.4.14	Encapsulation efficiency	45
3.4.15	Preparation of GA ₃ chitosan nanoparticles (CS).....	45
3.4.16	Preparation of GA ₃ chitosan-kefiran nanoparticles (CS/KEF).....	45
3.4.17	Preparation of GA ₃ alginate-chitosan nanoparticles (ALG/CS).....	45
3.4.18	Preparation of GA ₃ alginate-chitosan-kefiran nanoparticles (ALG/CS/KEF) .	46
3.4.19	Scanning electron microscopy (SEM).....	46
3.4.20	Statistical analysis	46
3.4.21	Release assays	46
3.4.22	Germination test	47
3.5	RESULTS AND DISCUSSION	48
3.5.1	Optimization of inoculum production.....	48
3.5.2	GA ₃ production by SSSF	48
3.5.3	Scale-up of GA ₃ production in BCR.....	49
3.5.4	Kinetics of GA ₃ production in STR.....	50
3.5.5	Encapsulation of GA ₃	51
3.5.5.1	GA ₃ beads	51
3.5.5.2	GA ₃ nanoparticles	52
3.5.6	Release assays	54
3.5.7	Germination assays	55
3.6	CONCLUSIONS AND PERSPECTIVES	58
4	GENERAL CONCLUSION	60
	REFERENCES	62
	APPENDIX 1 – PRODUCTION AND OPTIMIZATION OF INOCULUM.....	70
	APPENDIX 2 – PRODUCTION AND PURIFICATION OF GIBBERELLIC ACID	71
	APPENDIX 3 – BEADS AND NANOPARTICLES, WITH AND WITHOUT GIBBERELLIC ACID.....	72
	APPENDIX 4 – GERMINATION ASSAYS	73

1 INTRODUCTION

The plant growth hormones are signaling molecules that act in different ways due to their concentration and to the environmental conditions. These hormones are related to the growth and to development of plants (Vandenberghe et al., 2014). One of the most important groups of phytohormones is the gibberellins group. The gibberellic acid (GA_3) is responsible for the elongation of stem, flowering and seed's germination (Bömke e Tudzynski, 2009; Rodrigues et al., 2012). Therefore, these molecules present an enormous commercial interest for countries, such as Brazil, that have a significant part of your economy based on agro-industrial products.

Nowadays, GA_3 production is, majorly, done by the submerged fermentation. This technique has some disadvantages as low yield and high production cost that prevent the application of the biomolecule in extensive cultivars with low value (Rodrigues et al., 2012). Other fermentation techniques are being studied to resolve this problem. One of them is the submerged fermentation with solids in suspension or semisolid (Camara et al., 2020; Oliveira, 2012).

The semisolid fermentation allows the use of agro-industrial wastes as substrate for biomolecule production. So, the wastes were disposed causing an environmental impact, and now they can be used in bioprocess, so increasing the waste value and reducing the molecule's production cost (Pandey et al., 2000; Soccol e Vandenberghe, 2003).

Some agro-industrial by-products as citric pulp and soybean husk are being studied as substrate for GA_3 production using the fungi *Gibberella fujikuroi* (Oliveira et al., 2017; Rodrigues et al., 2009). The citric pulp is obtained by the orange juice production, containing the husk, seeds, and pulp, dried and pelletized (Pegoraro et al., 2012; Rodrigues, 2010). The extraction of soybean oil results in soybean husk as residue, which corresponds to 7-8% of the soybean weight, generally used in animal feeding (Restle et al., 2004; Rodrigues, 2010).

The following step is the downstream process, which is based on the separation and purification of GA_3 . In this step, the main idea is to obtain the higher yield of the purified biomolecule, once it has a high aggregate value (Shukla et al., 2003). The purified phytohormone has to be formulated to guarantee the activity stability and then can be commercialized (Kashyap et al., 2015; Liu et al. 2013). Nowadays, GA_3 is available in solid and liquid formulation.

In some recent researches, the encapsulation techniques and beads and nanoparticle production are described as an alternative formulation of GA₃ for controlled delivery in the environment (Blandón et al., 2016; Kashyap et al., 2015; Liu et al., 2013; Pereira et al., 2017a; Pereira et al., 2017b). Thereby, these systems are able to prolong the GA₃ action in plants, besides avoiding its degradation when exposed to certain conditions (Machado e Soccol, 2008; Preez et al., 1993).

The compounds most used in the formulation of beads, microparticle, and nanoparticle systems are biopolymers, as chitosan, pectin, alginate, kefiran, and cellulose (Blandón et al., 2016; Kashyap et al., 2015; Liu et al., 2013; Pereira et al., 2017a; Pereira et al., 2017b). The chitosan, which is obtained by the deacetylation of chitin, is used for GA₃ encapsulation due to its versatile properties and low cost (Liu et al., 2013; Pereira et al., 2017a). The alginate is an interest biopolymer, which is biodegradable and non-toxic due to its origin from brown alga. The exopolysaccharide extracted by the kefir grains is called kefiran and presents antimicrobial and good mechanical properties (Blandón et al., 2016; Kazazi et al., 2017; Piermaria et al., 2009). The biopolymer systems have to promote a controlled delivery of the bioactive, ensuring a higher time of action and protection of biomolecule (Venditti, 2019).

The main objective of this work was the production of GA₃ using the fungi *Gibberella fujikuroi* by semisolid fermentation of agro-industrial wastes (citric pulp and soybean husk), and its encapsulation in beads and nanoparticles to promote a controlled delivery in a cultivar, as an example soybean cultivar.

Chapter 1 is focused on the bibliographic review of the production and separation of GA₃ and its formulation related to new encapsulation techniques. In chapter 2, the experiments and their results were described, including the optimization of GA₃ production by semisolid fermentation, the encapsulation of GA₃ in different matrices, and the tests in soybean cultivar.

2 CHAPTER 1: STRATEGIES FOR PLANT GROWTH HORMONE'S (GIBBERELIC ACID) STABILITY - PRODUCTION, FORMULATION AND ENCAPSULATION USING NANOPARTICLES

2.1 ABSTRACT

Gibberellic acid (GA₃) is a plant growth regulator used to improve the growth and development of plants, which is mainly commercially produced by submerged fermentation using the fungi *Gibberella fujikuroi*. GA₃'s recovery and purification downstream process may occur with maximum yield avoiding undesirable costs. The regulator is commercialized in different types of liquid and solid (soluble powder, wettable powder, tablet, and soluble granule) formulations. However, some of these GA₃ formulations do not necessarily preserve its stability. Nanotechnology has introduced nanomaterials in diverse fields, being also applied in agriculture. To overcome the problem with GA₃'s stability, nanomaterials are being studied to produce delivery systems that can improve the hormone's stability and activity, preserving its properties. This is surely a field to explore, due to the high potential of these nanoparticles that can be applied in plant growth hormone delivery systems using different materials. This review is focused on the most recent advances in biotechnological production, separation/purification and formulation of GA₃ with a touch of the new possibilities that nanoparticles technologies bring for controlled delivery systems of this important plant hormone.

2.2 INTRODUCTION

Phytohormones are signaling molecules that act differently according to their concentration and to the environmental conditions in which plants are exposed. These phytohormones are generally related to plant's growth and development. The most relevant phytohormones are the gibberellins, auxins, such as indole-3-acetic acid, ethylene, cytokinins, and abscisic acid (Vandenberghe et al., 2014). Gibberellins are diterpenoid acids that promote stem elongation, flowering, seed germination, sexual development, dormancy, induction of enzyme activity, and senescence of fruits (Bömke and Tudzynski, 2009; Panchal e Desai, 2016; Tavares

et al., 2007). The existence of 136 gibberellins, nominated by GA₁ till GA₁₃₆, in plants, fungi, and bacteria has already been discovered. Among the 136 GAs, the most important are GA₁/GA₃ and GA₄/GA₇, because of their visible effects in plants, which certainly highlights their significant commercial importance (Bömke and Tudzynski, 2009; Rodrigues, 2010).

GA₃ is a tetracyclic dihydroxy-γ-lactonic that has two ethylene bonds and one free carboxylic acid group. It is responsible for promoting plant growth by causing stem elongation, flowering, germination, increase of cellular division and growth, and other positive physiologic effects. This phytohormone represents the primary product in the gibberellins biosynthesis pathway by the culture of *Gibberella fujikuroi*, mainly by SmF (Rios-Irube et al., 2016; Shukla et al., 2005).

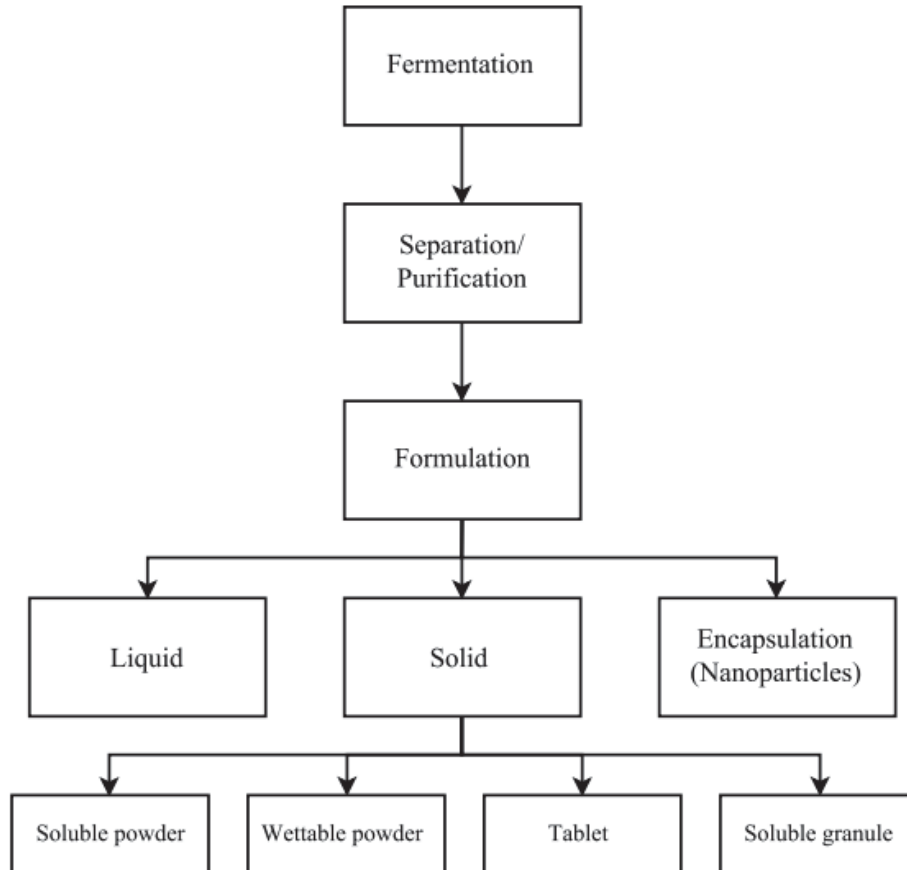
The steps of industrial GA₃ production are described in FIGURE 1. First, GA₃ is produced by SmF using microorganisms, such as *Gibberella fujikuroi*. Then the fermented broth can be separated and purified through adsorption, liquid-liquid extraction or membrane separation. Finally, in the formulation stage, the pure GA₃ is formulated with additives that promote its high stability. The product can be formulated in liquid or solid form (soluble powder, wettable powder, tablet, and soluble granule). The last reports present an alternative for the solid formulation that could be the encapsulation in nanoparticles' technique using different materials for the controlled delivery of phytohormones in the field (Kashyap et al., 2015; Liu et al., 2013; Pereira et al., 2017a; Pereira et al., 2017b).

Phytohormones require high stability when they are applied in the field due to the necessity of an extended action in plants, and their quick degradation when they are exposed to certain conditions of light and temperature. Furthermore, GA₃ in aqueous solutions can be decomposed quickly, and in alkaline conditions, it is modified with a significant decrease of its bioactivity (Machado and Soccol, 2008; Preez et al., 1993). These problems could be overcome by the use of materials that can promote delivery systems, which are able to maintain GA₃'s activity in plants for longer periods, preserving its properties and concomitantly promoting its controlled delivery.

Nanoparticles are nanoscale materials capable of encapsulating molecules such as drugs and other bioactive biomolecules such as GA₃, improving their stability. Some nanocarrier polymeric systems have been described in different forms such as

micelles, dendrimers, nanoparticles, nanogels, nanocapsules and vesicles, which are widely used as drug delivery systems.

FIGURE 1. Steps of GA₃ industrial production and formulation.



These nanostructures have some properties and often are made of polymers that respond to a stimulus or their environment, such as changes in temperature, pH, light, or redox potential, inducing dynamic and reversible changes useful for the controlled release of bioactive molecules (Venditti, 2019). For GA₃, some authors reported the use of these systems mainly based on chitosan. However, there are studies that use other materials in combination with chitosan (Kashyap et al., 2015; Khot et al., 2012; Liu et al., 2013; Nair et al., 2010; Pereira et al., 2017a; Pereira et al., 2017b).

2.3 GA₃ PRODUCTION BY FERMENTATION

GA₃ can be synthesized from chemical pathway or obtained by extraction from plants. However, these last two options are not economically viable. Currently,

GA₃ production, which have started in Japan, is carried out by SmF (Rios-Iribe et al., 2011; Rodrigues et al., 2012; Shukla et al., 2003). Other techniques for GA₃ production are being tested such as solid-state fermentation (SSF) and semisolid state fermentation (SSSF), or the also called submerged fermentation with solids in suspension, with the use of agro-industrial wastes as substrate (Kumar and Lonsane, 1990; Oliveira et al., 2017; Rangaswamy, 2012; Rodrigues et al., 2012; Tomasini et al., 1997).

SSF has attracted attention due to the major possibility of using agro-industrial sub-products, being successful in the secondary metabolites production (Rangaswamy, 2012; Rodrigues et al., 2009; Rodrigues et al., 2012; Shukla et al., 2003). This process is known to promote an environment with low water activity for the microorganism development, by using an insoluble material as a support for the fermentation process, which also serves as a source of nutrients. Therefore, SSF is an excellent technology to add value and promote the reuse of agro-industrial wastes (Rodrigues et al., 2012; Soccol and Vandenberghe, 2003; Tomasini et al., 1997).

SmF is a process that occurs in a liquid medium. The microorganisms are cultivated in the liquid where the nutrients are available, for biomass or metabolite production. GA₃ production by SmF process can be influenced by different physical factors: pH, temperature, aeration and agitation, and nutritional factors such as carbon and nitrogen sources (Rios-Iribe et al., 2016; Rodrigues et al., 2012; Shukla et al., 2003). Nevertheless, this technique is the most used by the industry due to its significant advantages, for example, SmF can be easily carried out, reducing space and costs (Rodrigues, 2010).

SSSF is a technique where the substrate is suspended in a liquid medium. Such as in SmF, homogeneity and facility of mass transfer can be observed in SSSF. The product recovery is more efficient than in SSF, with very good fungi growth. The SSSF is a simple technique with higher productivity and less energy consumption in comparison to SmF (Economou et al., 2010; Oliveira, 2012; Oliveira et al., 2017). GA₃ production using different techniques, which were described above, and alternative substrate are presented in TABLE 1.

TABLE 1. Recent GA₃ production using different alternative substrate and fermentation techniques.

Microorganism	Substrate	Fermentation	Bioreactor	Production	Time	Reference
<i>Fusarium moniliforme</i>	Citric pulp	SSF	Erlenmeyer flask (250 mL)	5.9 g/kg	72 h	Rodrigues et al., 2009
<i>Gibberella fujikuroi</i>	Glucose and corn oil	SmF	Continuo Stirrer Tank Bioreactor (7 L)	380 mg/L	288 h	Rios-Irube et al., 2011
<i>Fusarium moniliforme</i>	Jatropha seed cake	SSF	Erlenmeyer Flask	15 g/L	240 h	Rangaswamy, 2012
	Glucose	SmF	Erlenmeyer Flask	105 mg/g	96 h	
<i>Gibberella fujikuroi</i>	Corn oil	SmF	Batch Stirred Tank Bioreactor (7 L)	0.36 g/L	288 h	Rios-Irube et al., 2016
<i>Fusarium moniliforme</i>	Wheat bran	SmF with immobilized cells	Erlenmeyer flask (500 mL)	300 µg/mL	144 h	Panchal, 2016
<i>Fusarium moniliforme</i>	Solid citric pulp	SSF	Erlenmeyer flask (250 mL)	7.60 g/kg	144 h	
			Column bioreactor (0.25 L)	7.34 g/kg	120 h	
<i>Gibberella fujikuroi</i>	Aqueous extract of citric pulp	SmF	Erlenmeyer flask (250 mL)	236 mg/L	216 h	Oliveira et al., 2017
			Stirred tank reactor (10 L)	273 mg/L	96 h	
	Aqueous suspension of grinded citric pulp	SSF	Bubble column reactor (1.5 mL)	203 mg/L	216 h	
			Erlenmeyer flask (250 mL)	331 mg/L	240 h	
		Bubble column reactor (1.5 mL)	208 mg/L	216 h		

2.4 GA₃ SEPARATION AND PURIFICATION PROCESSES

The downstream process of GA₃ production begins with the separation and purification of the biomolecule from the fermented broth. Due to the high value of this molecule, it is imperative that this stage occurs with maximum yield. The step of purification will be different for each final commercial product. It is important to point out that some process conditions and solvents, which are employed in separation and purification, may not cause undesirable effects in plants (Probst, 1961). Some of the GA₃ commercial products must have a high purity and to attain this condition, several steps are necessary, which may increase difficulties and raise the final cost of the process (Shukla et al., 2003). First, the cells must be removed, which can be

done by filtration or centrifugation. The recovery of GA₃ from the fermented broth can be done by three techniques: adsorption, solvent extraction and separation through membranes. These methods can be followed by purification such as a repeated liquid-liquid partition. Finally, vacuum concentration can be used to concentrate the phytohormone and obtain a powder or crystalline product (Shukla et al., 2003).

One of the most used processes to recover GA₃ is based on three extractions with ethyl acetate. First, the fermentation broth goes through a filtration process. The filtered medium is acidified with HCl until pH is 2.0- 2.5, and then triple extraction with ethyl acetate is carried out. After extraction, it is necessary to remove the solvents and concentrate the GA₃, so the last step corresponds to a vacuum drying or a rotary evaporation, with the dissolution of the residue in methanol (Ates et al., 2006; Escamilla et al., 2000; Rios-Irribé et al., 2011; Rios-Irribé et al., 2016).

Berrios et al. (2010) described a technique for GA₃ extraction based on emulsion liquid membranes. In this study, they used a system containing water-in-oil emulsion composed by a KCl aqueous solution and SPAN 80 as a surfactant, which stabilizes n-heptane, using Aliquat 336 as a carrier. This system improves GA₃ extraction obtaining higher concentrations. This process is considered as an alternative to the multi-stage extraction process.

As an optional step, decolorization of the final product by using a decolorizing agent such as activated carbon, can also be conducted. Physical and chemical properties of porous activated carbon material were tested for their potential to be used for GA₃ clarification (Probst, 1961; Rodrigues, 2010).

2.5 GA₃ PRODUCT FORMULATION

In the market, there are different formulation types of GA₃ commercial products. Some examples of the hormone formulations are liquid, soluble powder, wettable powder, tablet and soluble granule (Devisetty et al., 2015). Different commercial products of GA₃ with different formulations and concentrations are available in the market and presented in TABLE 2.

The formulation step is essential because the formulation is related to the stability of the bioactive molecule. The choice of the correct formulation can increase the biomolecule's shelf life. Besides that, other characteristics are wanted for a plant growth regulator commercial formulation such as minimal damage to plants, lower

toxicity, and low environmental contamination. Due to these considerations, much research has been done to improve phytohormones formulations (Kashyap et al., 2015).

TABLE 2. GA₃ commercial products available in the market.

GA ₃ product	Formulation type	Concentration	Producers
Active BioGib		-	Agvance
GibGro® 20% Powder	Soluble powder	20% w/w	NuFarm Americas
N-Large™ 20 SP		20% w/w	StollerUSA
Quick-Dissolve™ Release		-	GoldBio
		10% w/w	Valent BioSciences
Activol 40 SG		40% w/w	Valent BioSciences
Berelex® 40 SG		40% w/w	Valent BioSciences
Express		400 g/kg	Ravensdown
ProGibb SG	Soluble granule	400 g/kg	Nufarm Americas
ProGibb® 40 SG		40% w/w	Valent BioSciences
RyzUp® 40 SG		40% w/w	Valent BioSciences
RyzUp SmartGrass		40% w/w	Valent BioSciences
GibGro®-20TB	Tablet	20% w/w	Nufarm Americas
GibbSTART MAX		200 g/L	Donaghys
PastureGibb™	Liquid	32 g/L	Orion Agriscience
ProGibb T&O		4% v/v	Valent BioSciences
RyzUp R 4 SL		4% v/v	Valent BioSciences

Source: (Agvance, 2017; Donaghys, 2017; GoldBio, 2017; Nufarm Americas, 2005; Nufarm Americas, 2017; Orion Agriscience, 2017; Ravensdown, 2017; Stoller Enterprises, 2013; Valent BioSciences, 2006; Valent BioSciences, 2009; Valent BioSciences, 2017).

2.5.1 GA₃ liquid formulation

For the application on crops, the gibberellins need a carrier such as a solvent system for easier application. These solution formulations have a low strength due to

the small concentrations of the hormone, which requires bigger packages, and consequently more space is necessary for the products' storage. In addition, it represents more costs for transportation and extra warehouse space under correct conditions (Devisetty et al., 2007; Devisetty et al., 2015). Therefore, liquid formulated products are more difficult to transport and have a short shelf life. Even so, liquid formulations are prepared by farmers with the dissolution of the plant hormone, GA₃, in an aqueous solution that is applied directly to cultivars using a spray. According to several studies about aqueous solutions GA₃ formulations, the molecule is usually hydrolyzed and decomposed. Because of that, it is always difficult to produce high stability liquid formulations (Kuhr, 1962). To avoid this problem, the use of water soluble solid additives has been studied. However, many of these substances can cause the loss GA₃ bioactivity (Pierre, 1961).

Due to GA₃ transformation in aqueous solutions, it is necessary to use other solvents. However, GA₃ has low solubility in these solvents, which makes it difficult to obtain high concentration formulations. Therefore, sometimes, to improve the solubility, solvents that have volatile organic compounds are used. Although, these solvents are not suitable for the environment, once they were related to the formation of ground-level ozone (Devisetty et al., 2015). Some solvents that are used to improve GA₃'s solubility and stability are toxic and flammable. Therefore, solvents such as isopropyl alcohol and methyl alcohol have many restrictions during manufacturing until warehousing (Devisetty et al., 2007). Solvents such as alcohols, especially methanol, are used in commercial products. But, methanol has properties that classify it as a flammable and toxic solvent. According to the Dangerous Goods Authorities, gibberellins' solutions must be classified and marked as flammable and poisonous, due to the presence of methanol in the liquid formulation (Killick et al., 2003). The commercial products in liquid formulations are usually dissolved in isopropanol, methanol or ethanol at a concentration around 4% (w/v) (Datta et al., 2006).

Studies have been done to develop liquid formulations for high GA₃ stability. A solution formulation that has low VOCs has already been patented. This patent describes a formulation that contains: at least one of the three gibberellins (GA₃, GA₄, and GA_{4/7}), polyethylene glycol as the primary compound, and a surfactant that can be anionic or non-ionic (Devisetty et al., 2015). Another similar patent claim exists a liquid solution formulation containing at least one gibberellin from the three cited

above, polyethylene glycol, and optional compounds as a non-aqueous co-solvent, surfactant, and minerals (Stoller and Sheth, 2016). These two patents have an environmental appeal with low VOCs, and consequently, very low emissions.

Killick et al. (2003) developed a system for plant growth formulation consisting of a lipophilic solvent system. The formulation is composed of one or more gibberellins, below 20% of the total of the weight; one or more lipophilic solvents, which represent the majority of its composition; a lipophilic alkaline coupling agent and emulsifiers to promote a homogeneous product.

Another liquid formulation study revealed the use of a solvent consisting of C_1 to C_4 esters of lactic acid and a C_2 to C_6 of polyhydric alcohols. The gibberellin is dissolved in this solvent forming a concentrated liquid formulation, which is based on gibberellin, lactate esters, and glycerol or propylene glycol. Some advantages of using this formulation are: no need of complex chemical substances, low cost ingredients, high stability, low volatility and high flash point (Datta et al., 2006).

A non-aqueous solution was described to improve plant growth hormone stability. The composition is based on the phytohormone, in polar and semi-polar organic solvents. As a result, the bioactivity of the molecule is preserved (Stoller and Sheth, 2016). A report described the study of GA_3 in urea solution. This combination is known to improve pasture production and leads to alkaline conditions. As an example, the commercial GA_3 product Express was employed in experiments with urea. Authors observed that after 24 hours no significantly GA_3 degradation occurred (Cotching et al., 2017a).

In the composition of the final product, there are also additives that can be used in GA_3 liquid formulations, which are classified according to their role. Some examples of four classes of additives are described in TABLE 3.

2.5.2 GA_3 solid formulation

To overcome all the problems associated with the liquid formulation, it is possible to work with solid formulations, which can be found in different forms as soluble powder, wettable powder, tablet, and soluble granule formulations. Another recent technique is encapsulation with the use of nanomaterials (Devisetty et al., 2015). Solid gibberellin formulations require a multistep process preparation comprising homogenization, mixing, extrusion, drying and/or other operations.

Moreover, for the solid formulation, the costs can increase due to the use of complex operations, more energy consumption, and use of additives/adjuvants (Datta et al., 2006; Devisetty et al., 2015).

TABLE 3. Different additives used in GA₃ liquid formulation.

Class	Examples
Surfactants	Carboxylates, sulfonates, alkylamides, ethoxylated alcohols, polyethylene, alkylaryl ethoxylates, polyalkyglycol esters, polysorbate
Antioxidants	Propyl gallate, ethoxyquin, butylated hydroxyanisole, butylated hydroxytoluene, tertiary butylhydroquinone
Minerals	Metal chlorides, metal sulfates, sodium or potassium salts, calcium, zinc, boron

Sources: (Devisetty et al., 2015; Stoller and Sheth, 2016; Wang et al., 2012)

Soluble powder formulations are produced after drying processes. In this case, GA₃ has the advantage of being dissolved when mixed with water forming a perfect solution. There is no need for agitation or mixing in a tank. For wettable powder formulations, the biomolecule (GA₃) is mixed with a dry carrier, such as mineral clay that is combined with other compounds to increase the suspension of the powder in water. The application of the produced suspension is carried out by spray. The suspension must be continuously agitated to avoid the production of insoluble particles (Devisetty et al., 2003; Devisetty et al., 2015).

Tablet is another type of formulation that can be applied in agriculture. It is a delivery system that has the correct dose for application. There are effervescent tablets that dissolve easily in water. The time for the complete dissolution depends on the type and size of the tablet; which may take from two to ten minutes. Tablet plant hormone formulations are not the most indicated for application in large-scale fields, due to the fact that they normally deliver only a small amount of GA₃, between 0.1 and 1 gram per tablet. In addition, tablets formulations are hygroscopic and expensive (Devisetty et al., 2003; Devisetty et al., 2007; Devisetty et al., 2015).

In the soluble granule formulation, the bioactive molecule is formulated as a dispersible granule. This type of formulation has low moisture, fast dissolution,

attrition resistance, and capacity of developing a suspension in aqueous solution. However, for a complete dispersion, this suspension must be agitated for a considerable time during the application (Devisetty et al., 2007; Geary et al., 1962; Killick et al., 2003).

One of the disadvantages of the solid formulation is the fact that the solid particles must have an accurate measurement. Additionally, depending on the type of application in crops, the use of solid formulations is more or less complicated. Another disadvantage of solid formulations is that complex chemicals are used. Some of them are petrochemically derived, and the U.S. Environment Protection Agency's has classified them as toxic ingredients for the use in the agricultural formulations (Datta et al., 2006).

A common problem of soluble powder, wettable powder, and soluble granule formulations is the necessity of tank-mixing for solubilization. These formulations can promote the production of dust, which can cause some health problems during their manipulation and application. The need for agitation increases the cost in addition to low yield recoveries and poor dispersion, leading to undesirable insoluble particles in the tank, forming cake, and the deposition of particles on plants that receive the spray application (Devisetty et al., 2003; Devisetty et al., 2007; Devisetty et al., 2015; Lloyd and Stuart, 1997). Studies have been conducted to check whether the recommended solubilization of commercial products is correct. Two commercial products were tested: Express and ProGibb SG. If the GA₃ product solubility is low, residues can be generated, leading to the formation of dust and caking, which may affect the plant hormone performance. In this case, authors reported that both commercial products showed correct solubility indication and mode of application (Cotching et al., 2017b).

For the preparation of solid formulations, such as soluble granule formulation, some types of components are needed: diluent, wetting agent, binder, anti-caking agent, antifoam, and surfactant. Some examples of these materials are described in TABLE 4.

The patent of Pierre (1961) describe a soluble granule formulation where GA₃ is mixed with diammonium phosphate and polyethylene glycol. These ingredients act as stabilizer and coating agents, respectively, offering a higher stability for the biomolecule. However, the formulation does not avoid the inactivation

of GA₃ in the presence of nitrates and urea. The patent of Rose (1962) reported the use of potassium salt to produce GA₃ crystals.

TABLE 4. Different types of ingredients for solid formulation and examples of chemicals.

Class	Chemicals
Diluent	Lactose monohydrate, sucrose, maltose, fructose, maltodextrin, corn syrup sugar, sorbitol, manitol
Wetting agente	Alkyl naphthalene sulfonates, phosphate esters, ethoxylated sorbitan esters, ethoxylated alcohols, polysorbate 20
Binder	Polyvinyl acetate, methyl cellulose, polyvinylpyrrolidone, maltodextrin, lignin, lecithin
Anti-caking agente	Silica gel, limestone
Antifoam	Polydimethylsiloxane
Surfactant	Ethoxylated fatty acid, glycol ester, ethoxylated alcohol, sorbitan fatty acid ester

Source: (Devisetty et al., 2003; Devisetty et al., 2007; Killick et al., 2003; Lloyd and Stuart, 1997; Pierre, 1961; Wang et al., 2012)

Devisetty et al. (2003) developed a water-soluble granular plant growth regulator formulation, comprising at least one gibberellin; at least one binder, such as polyvinylpyrrolidone; at least one disaccharide, such as lactose monohydrate; at least one surfactant as polyoxyethylene 20 monolaurate; and may have at least one additional component such as an anti-foaming agent, preservative, protectant, etc. This formulation was developed to improve the spray application of GA₃, so some properties such as facility of measurement, quick dissolution, and being dust-free for handling were described.

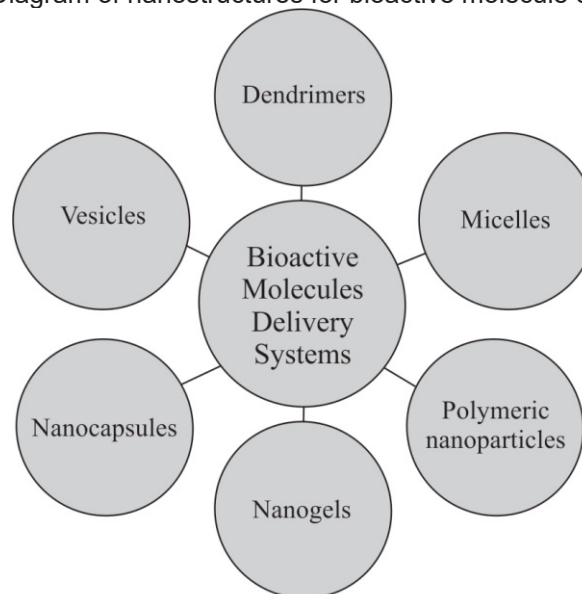
2.6 ENCAPSULATION METHODS FOR GA₃ CONTROLLED RELEASE

Nanotechnology is based on the use of nanomaterials, which consist of particles with less than 100 nm. The successful combination of nanotechnology and biotechnology has introduced nanomaterials in diverse fields, leading to new

applications. Nanomaterials have several uses as in wastewater treatment, medicine, and water purification (Khot et al., 2012; Nair et al., 2010). Currently, several studies can be found on the use of nanoparticles, which act as delivery systems for the controlled release of chemicals, such as pesticides, fertilizers and herbicides, micronutrients, genetic material for plant transformation, such DNA and RNA, and plant growth regulators (Kashyap et al., 2015).

Nowadays, the use of nanoparticles as delivery systems of bioactive molecules is widely studied. These systems provide a controlled release of these molecules, which avoids repeated application and decreases their costs. Polymeric materials are most often used for the delivery system production. The polymers should respond to a stimulus such as variation in temperature, pH, light, and redox potential to control the release of the bioactive molecules. These polymers can form several nanostructures: vesicles, dendrimers, micelles, polymeric nanoparticles, nanogels, and nanocapsules, as it can be seen on FIGURE 2 (Venditti, 2019).

FIGURE 2. Diagram of nanostructures for bioactive molecule delivery systems.



Vesicles are nanostructures used carriers that have great properties such as nanoscale size, high surface-to-volume ratio, and physic-chemical characteristics of interest. The most important characteristic of vesicles is the stimuli-responsive, which affects the release of bioactive molecules (Marianecchi et al., 2016; Venditti, 2019). Nanostructures called dendrimers have a highly branched morphology with a central core. This structure is useful for attaching a bioactive compound. The dendrimers'

properties, such as its three-dimensional structure, surface with functional group, and versatility, facilitate the use of this nanostructure as a carrier for drugs. These properties can be interesting for the encapsulation of other bioactive molecules (Najlah and D'Emanuele, 2006; Venditti, 2019).

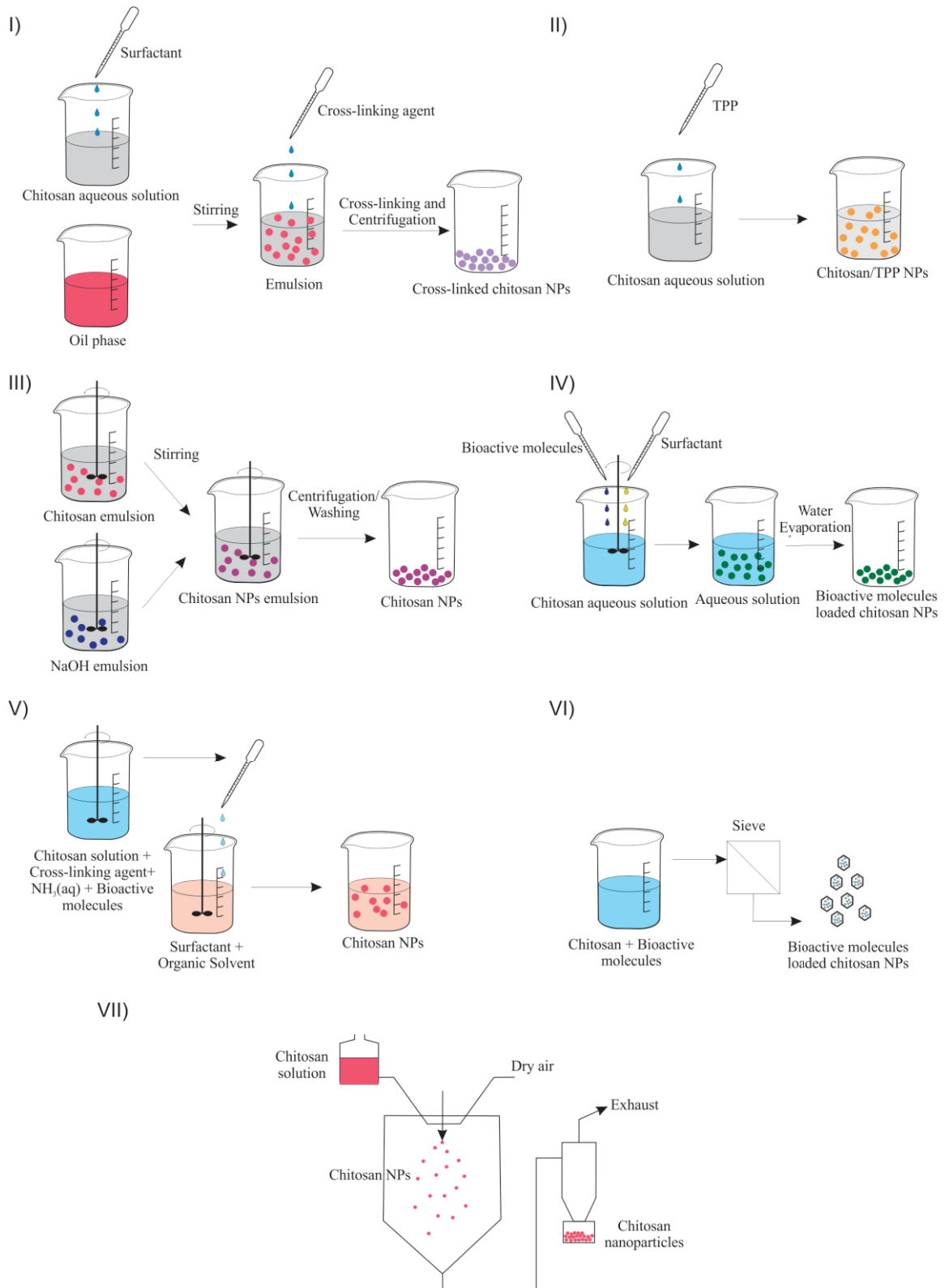
Polymeric micelles are amphiphilic polymeric molecules that in aqueous medium are capable of forming core-shell structures. Due to their multifunctionality, micelles can be applied as carriers for other molecules besides drugs. The micelles also have the property of stimuli responsiveness. However, polymeric micelles are instable. One strategy described to overcome this problem is the cross-linking approach (Cong et al., 2018; Venditti, 2019).

Polymeric nanoparticles are used for encapsulation of diverse bioactive compounds. The polymers used to produce nanoparticles are biodegradable such as chitosan, alginate and albumin. These biodegradable materials are normally used because of their bioavailability, control release, great encapsulation efficiency, and low toxicity (Kumari et al., 2010; Venditti, 2019).

Nanogel is a polymeric nanocarrier composed of hydrogel with nanoparticles. Therefore, this composition guarantees the characteristics of both materials. The production of nanogels can be done using a physical or chemical approach. The interactions between polymer chains lead to the formation of nanogels. Due to their physical interactions the nanogels are able to encapsulate other bioactive compounds besides drugs (Zhang et al., 2016). The nanocapsule structures are based on a spherical hollow structure in which the bioactive compound is trapped in a cavity surrounded by a polymeric membrane. The polymers used to prepare the structure are biodegradable polymers that can be natural or synthetic. In the cavity the bioactive molecule can be in solid or liquid form or as a molecular dispersion. The six most used methods for nanocapsules' preparation are: nanoprecipitation, emulsion-diffusion, double emulsification, emulsion-coacervation, polymer-coating, and layer-by-layer (Mora-Huertas et al., 2010; Venditti, 2019).

For the production of encapsulated bioactive compounds, some strategies can be employed such as ionotropic gelation, emulsion-droplet coalescence, emulsion cross-linking, reverse micellar method, precipitation, sieving method, and spray-drying method. Each of these techniques has pros and cons. A simple scheme of these methods using chitosan as the nanomaterial is shown in FIGURE 3 (Kashyap et al., 2015).

FIGURE 3. Methods of encapsulation based on chitosan used as the nanomaterial: I) Emulsion cross-linking; II) Emulsion-droplet coalescence; III) Ionotropic gelation; IV) Precipitation; V) Reverse micelles; VI) Sieving; VII) Spray drying. NPs: nanoparticles.



Ionotropic gelation is a simple and non-aggressive technique, and the degradation kinetic is controllable. This method decreases the toxic chemical effects,

because there is no chemical cross-linking in the method. However, the properties of the material used for encapsulation, such as chitosan, can affect the release of the active compound (Kashyap et al., 2015).

The strategy of emulsion-droplet coalescence has the characteristic of carrying more of the active ingredient, and the particle size can be small. However, depending on the material used for the nanoparticles formulation, the particle size varies. For example, chitosan has a different degree of deacetylation; when this degree is lower the particle size increases. With this variation of particle size, the amount of active molecules inside the particle can easily oscillate (Kashyap et al., 2015). The particle size is one of the most critical properties in encapsulation. The emulsion cross-linking method can control this. Besides that, the method promotes a higher bioavailability of the active compound. However, it is a complicated process. The active ingredient can react with the cross-linking agent. The reverse micellar method is not a simple technique, but leads to a thermodynamically stable particle with the appropriate polydispersity (Kashyap et al., 2015).

A technique for an efficient release control with a high distribution of particle size is precipitation. The important point is that precipitation does not use toxic organic solvents; however, it does not entirely protect the biomolecule against nucleases activity. The sieving method produces particles with irregular shape, but it can be easily scaled-up and process.

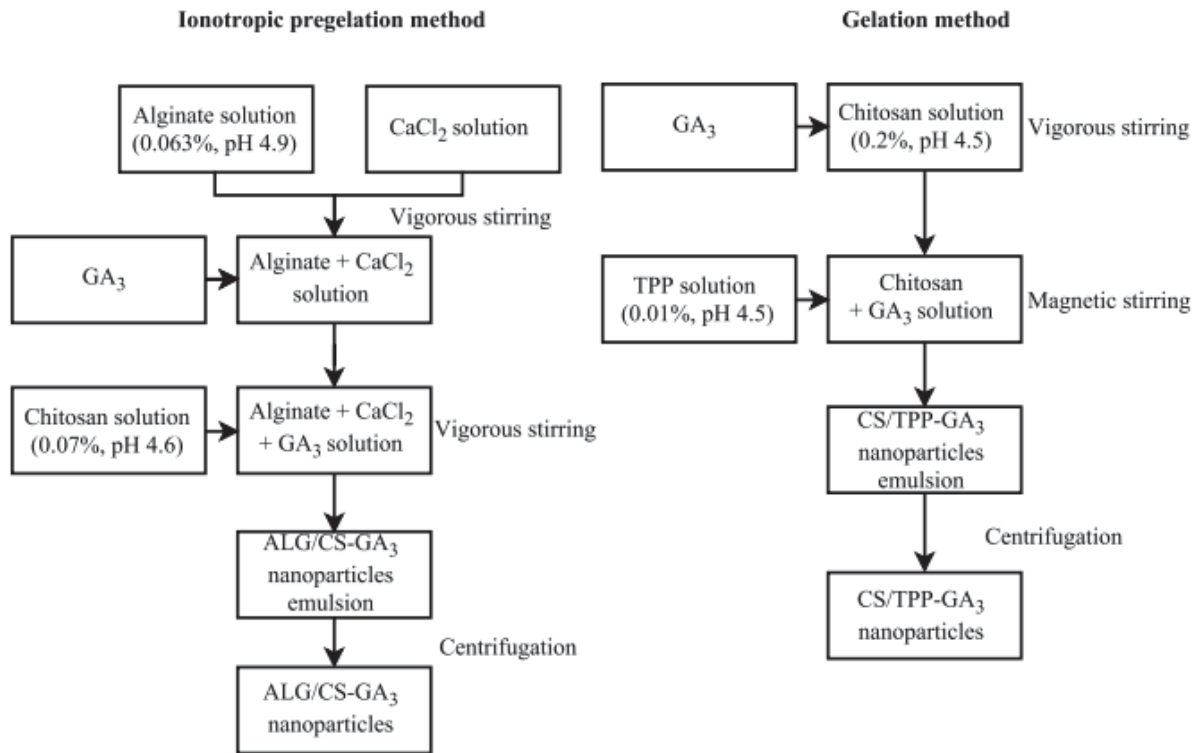
Finally, the spray-drying method shows an excellent stability of the active ingredient and release control. This technique is very often used for powder formulation, but there are many variables during the procedure that can change the particle size (Kashyap et al., 2015).

A report by Pereira et al. (2017b) describes two different processes for the preparation of alginate/chitosan and chitosan/tripolyphosphate nanoparticles, which can be used as a carrier system for GA₃. In FIGURE 4 it is possible to observe the procedures steps. For the preparation of alginate and chitosan (ALG/CS) nanoparticles, an ionotropic pre-gelation method was described by Sarmiento et al. (2006). The CaCl₂ solution is slowly added to ALG solution under high agitation. The active ingredient, GA₃, is attached to the Chitosan solution that is prepared with the addition of acetic acid. The nanoparticles are then formed and to recover the ALG/CS-GA₃ nanoparticles a centrifugation is carried out. The same method, with some modifications, was used to produce poly(γ -glutamic acid) and chitosan (γ -

PGA/CS) nanoparticles. Instead of alginate solution the procedure was conducted with a γ -PGA solution (Pereira et al., 2017a; Pereira et al., 2017b).

The process used for the production of chitosan and tripolyphosphate (CS/TPP) nanoparticles was the gelation method (FIGURE 4). This method is based on the addition of the GA_3 , active molecule, in the chitosan solution. Then after the dissolution of GA_3 , the TPP solution is added. The two steps occur under stirring, and then a centrifugation step is needed to recover the CS/TPP- GA_3 nanoparticles (Pereira et al., 2017b).

FIGURE 4. The ionotropic pregelation and gelation methods for GA_3 encapsulation.



ALG/CS: alginate and chitosan; $CaCl_2$: calcium chloride; CS/TPP: chitosan and tripolyphosphate; GA_3 : gibberellic acid; TPP: tripolyphosphate.

Physical and chemical characterization of ALG/CS and CS/TPP nanoparticles was carried out, where some parameters were analyzed: size distribution, polydispersity index, zeta potential, and encapsulation efficiency. Besides that, the profiles of GA_3 release under different conditions of pH and temperature were also determined. The ALG/CS and CS/TPP system presented, respectively, 100% and 90% encapsulation efficiency. For both systems, the nanoparticles were stable for 60 days. The study showed that either the ALG/CS- GA_3

or the CS/TPP-GA₃ system can be applied in agriculture due to the fact that these systems have different release mechanisms, so their effect on plants' responses is different. The ALG/CS-GA₃ nanoparticles showed a faster release, so they are able to result in greater short-term effect. In the case of CS/TPP-GA₃ nanoparticles, there is a slow release of the hormone, which is due to stronger interactions that promotes greater effects after long periods (Pereira et al., 2017b).

γ -PGA/CS nanoparticles were characterized by their size, polydispersity, zeta potential, and encapsulation efficiency. For this nanoparticle system, the encapsulation efficiency reached 61% with 58% of GA₃ prolonged release after 48 hours, and achieved protection against degradation. The study showed that the γ -PGA/CS-GA₃ nanoparticles have a considerable potential for application in agriculture (Pereira et al., 2017a).

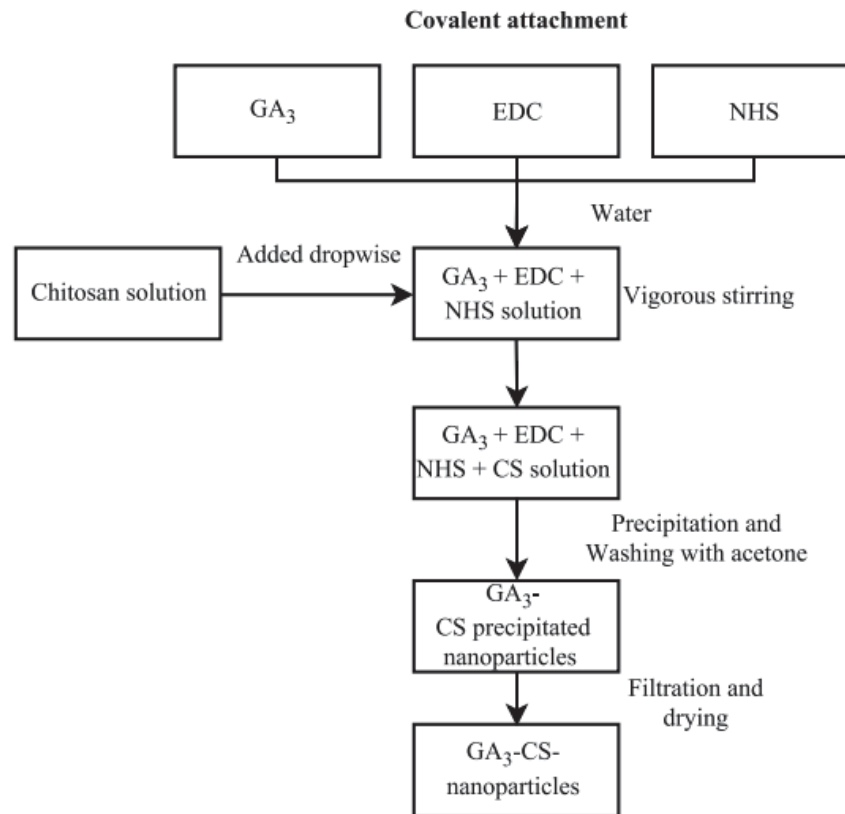
Liu et al. (2013) reported a technique for the production of GA₃-CS nanoparticles, which is the covalent attachment (FIGURE 5). This method consists of the dissolution of three ingredients in water, the plant growth hormone (GA₃), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS). After that, a chitosan solution is added dropwise to the solution under stirring. The pH is adjusted and the solution is kept at 4°C during a day. Then the precipitation occurs and nanoparticles are washed with acetone. The nanoparticles are recovered by filtration and dried in an oven (Liu et al., 2013).

GA₃-CS nanoparticles were analyzed by Fourier transform infrared spectroscopy (FT-IR), ultraviolet spectrophotometer, and thermal gravimetric analysis. The controlled release of GA₃ was analyzed in different conditions of pH, temperature, and UV irradiation. The study showed a modification degree of more than 60% w/w in the CS conjugate. The release assay revealed that these nanoparticles are able to protect the phytohormone against quick degradation, especially photo- and thermal-degradation. Besides that, the GA₃-CS nanoparticles were able to maintain a constant GA₃ concentration for 60 days under different pH conditions (Liu et al., 2013).

Some important properties of carrier materials used for controlled release of some formulations in agriculture are biodegradability and non-toxicity. Promising materials used as carrier systems are natural polymers, such as pectin, starch, chitosan, cellulose, alginate, cyclodextrin, dextran, and γ -PGA polymer. The

controlled release of the active ingredient improves the action of the active molecule (Kashyap et al., 2015; Liu et al., 2013; Pereira et al., 2017a; Pereira et al., 2017b).

FIGURE 5. The covalent attachment technique for GA₃-CS-nanoparticles formation.



CS: chitosan; EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; GA₃: gibberellic acid; NHS: N-hydroxysuccinimide.

The phytotoxicity of the nanomaterials should be studied very carefully to avoid adverse effects on plants. The toxicity for human and the environment should be also considered. In this field, the use of nanosilver in agriculture was already discussed. These nanoparticles are used due to their antifungal and antibacterial activity avoiding the growth of phytopathogens. However, a chemical change and silver oxides production or ions occur when the particle is in powder. To overcome this problem, the use of a biocompatible polyvinyl pyrrole was reported to coat the nanoparticles. The use of biocompatible coatings can decrease the toxicity of some nanomaterials, so the applications of them in plants would be more significant (Campos et al., 2014; Khot et al., 2012; Nair et al. 2010).

To overcome the problem of GA₃'s low stability, which occurs under some conditions of light and temperature, the encapsulation technique can be used,

providing a carrier system that enhances the biomolecule stability (Kashyap et al., 2015). Some carrier systems were developed for GA₃. One of them uses chitosan and an inorganic magnesium-aluminum layered double-hydroxide material. This nanosystem shows a slow GA₃ degradation. Another study used the biopolymer γ -PGA to produce a carrier system with chitosan for GA₃ delivery that presented some properties such as hydrophilicity, non-toxicity, biodegradability, and edibility, promoting the use of this polymer in food industry. Chitosan is the most used nanomaterial for GA₃ encapsulation. This molecule is a polysaccharide produced by deacetylation of chitin and it is used to develop carrier systems due to its economical and versatile characteristics. This natural polymer has the advantage of forming gel and beads formulation (Liu et al., 2013; Pereira et al., 2017a).

Pereira et al. (2017b) worked with two polymer combinations as nanoparticles materials: alginate and chitosan and chitosan and tripolyphosphate. Alginate is a biopolymer obtained from brown algae and has some advantages such as biodegradability, non-toxicity, and biocompatibility. These nanoparticles act as carriers promoting a controlled release system, which also maintain the biomolecule stability. Some matrices used as a delivery system of GA₃ and their characteristics when employed in nanoencapsulation are presented in TABLE 5.

TABLE 5. Matrices employed as GA₃ delivery system and their characteristics.

Matrices	Characteristics
Chitosan-alginate	Slow initial GA ₃ release, strong effects on leaf development and level of carotenoids
Chitosan- γ -PGA	Sustained release, GA ₃ protection against degradation, accelerate germination within 24 h after treatment
Chitosan-tripolyphosphate	Stronger interaction with GA ₃ , slow release high activity after long period of time
Chitosan	Good water solubility, protects GA ₃ from quick degradation, especially from photo and thermal degradation

Sources: (Liu et al., 2013; Pereira et al., 2017a; Pereira et al., 2017b)

2.7 CONCLUSIONS

GA₃, the important plant hormone, used in different cultivars all over the world, is mainly produced by SmF of the fungus *Gibberella fujikuroi*. After separation and purification, the hormone may be stabilized through formulation. Commercial formulations of GA₃ are in liquid and solid forms whose mode of application and stability may vary according to the chosen system. The common commercial solid formulations are soluble powder, wettable powder, tablet, and soluble granule. These formulations may present the risk of toxicity and cause residual deposition of dust. However, the use of nanotechnology to produce nanoparticles containing bioactive molecules can improve molecule's stability and activity of the hormone. For the controlled release of GA₃ only a few studies were described till now, mainly based on the use of the chitosan. There is certainly a large opportunity of testing new GA₃ controlled delivery systems using other nanomaterials in conjunction with chitosan or not, thereby amplifying the possibility of using this hormone in agriculture more efficiently, with higher stability and lower costs.

3 CHAPTER 2. STUDY OF DIFFERENT SYSTEMS FOR CONTROLLED DELIVERY OF GIBBERELIC ACID PRODUCED IN SUBMERGED FERMENTATION BY *Gibberella Fujikuroi*

3.1 ABSTRACT

Gibberellic acid (GA_3) is a plant growth regulator (PGR) used to improve growth and development of plants. GA_3 was produced by submerged fermentation using citric pulp (CP) and soybean husks (SH). The PGR was purified and encapsulated in different systems. The influence of controlled release of free and encapsulated GA_3 on soybean seeds' germination and root length was studied. GA_3 production reached 217 mg/L in STR using a medium composed of 3.5 % (w/v) CP, 1.5 % (w/v) SH, supplied with 180 g/L sucrose and 4 g/L urea. Alginate with GA_3 (ALG- GA_3), and alginate and kefiran with GA_3 (ALG/KEF- GA_3) beads, and chitosan with GA_3 (CS- GA_3) microparticles were produced. It was possible to observe an impact on seeds' germination and root length caused by the beads and nanoparticles, with and without GA_3 . The ALG- GA_3 system was able to achieve 100% of germination, against 40% obtained at the control system. The higher root length was observed in the treatment that the ALG/KEF- GA_3 bead system was placed in vermiculite with the seeds. The positive effect on cultivar leads to a gain of productivity that impacts the countries with an economy based on the agro-industrial.

3.2 INTRODUCTION

Phytohormones are signaling molecules that act differently according to their concentration and the environmental conditions in which plants are exposed. Gibberellins, one of the most important group of phytohormones, are diterpenoid acids that act as a plant growth and development regulator (Vandenberghe et al., 2014).

Nowadays, 136 gibberellins have been discovered, nominated by GA_1 till GA_{136} . The most important are GA_1/GA_3 and GA_4/GA_7 , due to their positive physiologic effects in plants (Bömke and Tudzynski, 2009; Rodrigues, 2010). GA_3 is a tetracyclic dihydroxy- γ -lactonic that has two ethylene bonds and one free carboxylic acid group. GA_3 is known to promote plant growth by causing stem

elongation, flowering, seed germination, dormancy, increase of cellular division and growth, which emphasize their significant commercial importance in places where the economy is based on the agroindustry (Rodrigues et al., 2012; Soccol and Vandenberghe, 2003).

This PGR represents the primary product in the gibberellins biosynthesis pathway by the culture of *Gibberella fujikuroi*, mainly by submerged fermentation (SmF) (Rios-Iribe et al., 2016; Shukla et al., 2005). There are other fungi, besides *G. fujikuroi*, and bacteria that are capable of producing GA₃. Besides that, GA₃ can be synthesized from chemical pathway or obtained by extraction from plants. However, due to the low production yield of the phytohormone, they are commercially infeasible (Ates et al., 2006; Bömke and Tudzynski, 2009; Escamilla S et al., 2000; Shukla et al., 2003).

Techniques such as solid-state fermentation (SSF) and semisolid state fermentation (SSSF), or the also called submerged fermentation with solids in suspension, with the use of agro-industrial wastes as substrate have been tested with the use of alternative substrate with reduced costs (Gökdere and Ateş, 2014; Oliveira et al., 2017; Páramo et al., 2004; Rangaswamy, 2012; Rios-Iribe et al., 2011; Rodrigues et al., 2012; Shukla et al., 2005).

SSSF is a technique where the solid waste substrate is suspended in a liquid medium without the need of a previous chemical or enzymatic treatment. The product recovery is more efficient than in SSF, and it promotes very satisfactory fungi growth. SSSF is a simple technique with high productivity and less energy consumption in comparison to SmF (Economou et al., 2010; Oliveira, 2012; Oliveira et al., 2017).

Agro-industrial wastes represent a serious problem for countries such as Brazil that has one of the major economies based on agriculture. Hence, these countries are responsible for a huge agro-industrial waste disposal that causes serious environmental concerns (Rodrigues, 2010; Soccol and Vandenberghe, 2003). In the last few years, some of the by-products have been used as an alternative substrate in fermentation, adding value to these wastes and, in parallel, solving environmental problems and reducing processes' costs (Oliveira, 2012; Pandey et al., 2000; Soccol and Vandenberghe, 2003).

In some recent researches, agro-industrial by-products such as citric pulp and soybean husk were used as substrate to produce gibberellic acid by *Gibberella fujikuroi* (Rodrigues, 2010). CP is a by-product from the orange juice production,

which is composed of orange husk, seeds and pulp that are dried and pelletized. It represents almost 50% of the fruit weight and it is used in the cattle rising as supplement for animal feed (Pegoraro et al., 2012; Rodrigues, 2010). According to IBGE, in 2016 the orange production in Brazil reached 17 millions of tons (Embrapa, 2017). SH is obtained as a by-product of the soy oil extraction; it corresponds to 7-8% of the soybean weight and it is also used for animal feed (Restle et al., 2004; Rodrigues, 2010). In the harvest of 2017/2018, Brazil produced almost 117 millions of tons of soybean (Embrapa, 2018).

Because of the high value of GA₃, it is imperative that the downstream process, which consists of two steps, the separation and purification of the GA₃ from the fermented broth, is conducted with maximum yield. The recovery of GA₃ from fermented broth can be done by three techniques: adsorption, solvent extraction and separation through membranes (Shukla et al., 2003). It is important to point out that some process conditions and solvents, which are employed in separation and purification, may cause undesirable effects in plants.

Before commercialization of GA₃, the pure PGR must be formulated, that is essential for bioactive molecule's stability (Kashyap et al., 2015; Liu et al., 2013). The correct formulation can increase the biomolecule's shelf life. Other characteristics are wanted for a PGR commercial formulation such as minimal damage to plants, lower toxicity, and low environmental contamination (Kashyap et al., 2015). The product can be found in the market in two forms: liquid or solid.

Liquid formulated products are more difficult to transport, require bigger packages and have shorter shelf life (Devisetty et al., 2007; Devisetty et al., 2015). As GA₃ is usually hydrolyzed and decomposed in aqueous solutions formulations, it is always difficult to obtain high stability liquid formulations (Kuhr, 1962).

GA₃'s solid formulation is an alternative to overcome all the problems associated with instability. Solid formulations are found in different forms as soluble powder, wettable powder, tablet, and soluble granule formulations (Devisetty et al., 2015; Kashyap et al., 2015; Liu et al., 2013). The production costs can increase due to the use of complex operations, more energy consumption, and use of additives/adjuvants (Datta et al., 2006; Devisetty et al., 2015).

Recent reports present an alternative for GA₃'s solid formulation that could be its encapsulation in nanoparticles using different materials for the controlled delivery of the PGR in the field (Kashyap et al., 2015; Liu et al., 2013; Pereira et al.,

2017a; Pereira et al., 2017b). The use of these systems can extend the action of GA₃ in plants, avoid its quick degradation under certain conditions of light and temperature, preserving its properties (Machado and Soccol, 2008; Preez et al., 1993).

Due to the successful combination of nanotechnology and biotechnology some nanomaterials were introduced in diverse fields promoting new applications (Khot et al., 2012; Nair et al., 2010). Different nanocarrier polymeric systems, which are widely used as drug delivery systems, have been described (Venditti, 2019). The materials used to produce nanoparticles are biodegradable and natural polymers, such as pectin, starch, chitosan, cellulose, alginate, dextran, kefiran and γ -PGA polymer (Blandón et al., 2016; Kashyap et al., 2015; Liu et al., 2013; Pereira et al., 2017a; Pereira et al., 2017b).

Chitosan is the most used nanomaterial for GA₃ encapsulation. This polysaccharide is produced by deacetylation of chitin and it is used to develop carrier systems due to its economical and versatile characteristics (Liu et al., 2013; Pereira et al., 2017a) Alginate is a biopolymer obtained from brown algae and has some advantages such as biodegradability, non-toxicity, and biocompatibility. Kefiran is an exopolysaccharide containing glucose and galactose that is obtained from kefir grains. Recently, some activities such antibacterial, antifungal, antioxidant and antitumor have been reported for kefiran. This water-soluble polysaccharide is able to form gels at low temperature and films with very good mechanical properties (Blandón et al., 2016; Dailin et al., 2014; Esnaashari et al., 2014; Kazazi et al., 2017; Piermaria et al., 2009).

Biodegradable materials are normally used to produce capsules or nanocapsules for bioactive compounds delivery because of their bioavailability, controlled release, great encapsulation efficiency, and low toxicity (Kumari et al., 2010; Venditti, 2019).

3.3 OBJECTIVES

The main objective of this work is the production of GA₃ by *Gibberella fujikuroi* NRRL 2278 in SSSF using citric pulp and soybean husks as substrate, the encapsulation in beads and nanoparticles for its controlled release using soybean as example of cultivar.

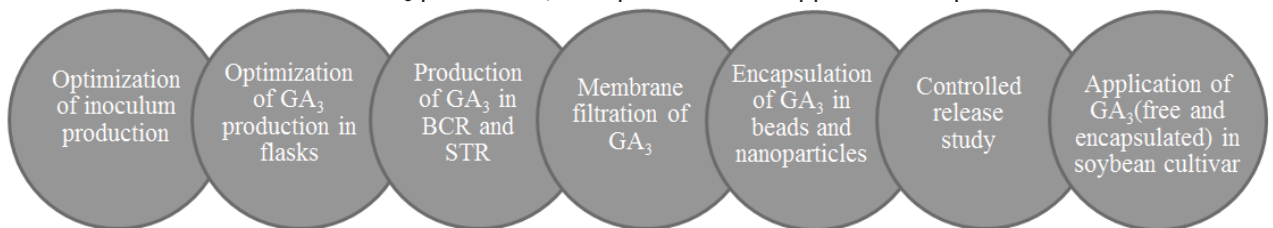
3.3.1 Specific objectives

- To optimize inoculum production conditions for GA₃ production;
- To optimize GA₃ production in flasks;
- To produce GA₃ by SSSF in BCR and STR;
- To separate and concentrate GA₃ by micro and ultrafiltration;
- To encapsulate GA₃ in beads and nanoparticles;
- To study standard GA₃'s controlled release;
- To test standard and clarified GA₃ (free and encapsulated) in soybean cultivar.

3.4 MATERIAL AND METHODS

Experimental essays were conducted according to the steps presented in FIGURE 6.

FIGURE 6. GA₃ production, encapsulation and application steps.



3.4.1 Microorganism

Gibberella fujikuroi NRRL 2278 from the strain bank of Bioprocess Engineering and Biotechnology Laboratory of Federal University of Paraná. The strain was stored in PDA slants at 4°C and subcultured every 3 months.

3.4.2 Substrates

CP and SH, were obtained from Coalma Animal Nutrition- SP and IMCOPA-Colombo/PR, respectively. CP and SH were dried at 40 °C for 8 hours, ground in a mill, classified to a particle size smaller than 5 mm, and then correctly stored.

3.4.3 Inoculum preparation

Citric Pulp and Soybean Husks Aqueous Extract (CP/SHAE) medium, at a concentration of 10% (w/v), containing 70% of CP and 30% of SH, was pre-treated at 100°C for 30 minutes. CP/SHAE was filtered to separate the solids (Oliveira, 2012; Rodrigues, 2010). 50 mL of the extract, which was diluted in deionized water (1:3), were added to a 250 mL Erlenmeyer flask. Flasks were sterilized at 121°C during 15 minutes. After cooled, the medium was inoculated and incubated in a shaker for 4 days at 29°C, 120 rpm.

3.4.4 Optimization of inoculum production

The optimization of inoculum production comprised the study of different levels of two factors, urea (0 to 16 g/L) and sucrose (from 0 to 207 g/L), which were added as supplements to CP/SHAE medium. A CCRD experimental design factors with triplicate of the central point was employed. Biomass production was determined by the dry weight method.

3.4.5 Optimization of GA₃ production by semisolid fermentation (SSSF)

GA₃ production by SSSF was carried out using a fermentation medium composed of 3.5% (w/v) of CP and 5% (w/v) of SH. 250 mL Erlenmeyer flasks, containing 50 mL of the suspension, were sterilized for 15 min at 121°C. After cooled, flasks were then inoculated (10% (v/v)) and incubated at 29°C, 120 rpm. A study of the effect of the addition of supplementary sucrose and urea concentrations to the fermentation medium was done. Fermentation essays were carried out during 5 days.

3.4.6 GA₃ production by SSSF in bubble column reactor (BCR)

GA₃ production was conducted in a 1.5 L BCR, which was projected in the Bioprocess Engineering and Biotechnology Laboratory of UFPR. Filled with 1 L of the suspension composed of 3.5 % (w/v) CP, 1.5 % (w/v) SH, 180 g/L sucrose and 4 g/L urea. The BCRs were sterilized by 15 min at 121°C and inoculated with an inoculum

rate of 10% (v/v). Each reactor was maintained at different aeration rates (1, 3 and 5 vvm). Antifoam was added when necessary. Fermentation essays were carried out for 7 days at 29°C. Samples were withdrawn each 24 hours, after the 4th day, to analyze the GA₃ production.

3.4.7 GA₃ production by SSSF in stirred tank reactor (STR)

The STR (Inceltech, LH – SGI, Set 2M), with the total volume of 2 L, was filled with 1 L of the medium composed of 3.5 % (w/v) CP, 1.5 % (w/v) SH, 180 g/L sucrose and 4 g/L urea. The reactor was sterilized during 15 min at 121°C. The STR was inoculated at a rate of 10% (v/v). STR was maintained at 29°C, with an aeration rate of 3 vvm, and 500 rpm. The antifoam was added when necessary. Fermentations assays were carried out during 5 days. Samples were withdrawn each 24 hours after for GA₃ production analysis.

3.4.8 Analysis of GA₃ production

After fermentation, the fermented broth was filtered and clarified using Carrez solution (Lu et al., 1995; Machado et al., 2002). 15 mL of the broth were added of 1 mL of zinc acetate (30% w/v), 1 mL of potassium ferrocyanide (15% w/v), 5 mL of ethanol and 3 mL of distilled water. The solution was stirred and then filtered. GA₃ was quantified by spectrophotometry method. The clarified extract was acidified with 30% HCl solution and kept for 1 hour at 20°C. The absorbance was then determined at 254 nm (Holbrook et al., 1961).

3.4.9 Analysis of reducing sugars

The concentration of reducing sugars of the fermentation medium was quantified by Somogyi-Nelson method (Nelson, 1944). The absorbance was determined at 535 nm.

3.4.10 Membrane Separation and purification of GA₃

GA₃' fermentation broth was subjected to centrifugation, for 10 min at 2000 rpm, and a vacuum filtration using Whatman filter paper. Then, the filtrate passed through microfiltration (pore size of 0.2 µm) and two ultrafiltration (100 kDa and 10 kDa) steps using a VivaFlow 200 (Sartorius Stedim, Germany) crossflow filtration system.

3.4.11 Kefiran (KEF) production and extraction

Kefir grains cultivated in cheese whey at 30°C during 10 hours were suspended in water at 100°C for 30 min. Then, the suspension was centrifuged at 4000 rpm during 20 min at room temperature. The precipitate was discarded. The supernatant was precipitated with cold ethanol, -20°C (1:4). After that, the suspension was centrifuged at 4000 rpm for 20 min at 4°C and the precipitate was dissolved in hot water. The precipitation was repeated twice and KEF was freeze-dried (Blandón et al., 2016).

3.4.12 Preparation of GA₃ alginate beads (ALG)

An aqueous solution of 2% (w/v) sodium alginate (Sigma Alldrich) was prepared and added of 200 mg/L GA₃. The formation of beads was based on the external gelation method. ALG solution with GA₃ was added dropwise into a 500 mM CaCl₂ solution under stirring. The beads were washed with water twice and stored at 4°C. Beads were also prepared without GA₃ (Blandón et al., 2016).

3.4.13 Preparation of GA₃ alginate-kefiran beads (ALG/KEF)

A 2% (w/v) KEF solution was prepared in hot water and 2% (w/v) ALG solution was prepared in citrate buffer (pH 5) in ice water bath. Both solutions were mixed at a ratio of 1:1 so as to have a final concentration of 1% (w/v) of each polymer. Then, the standard GA₃ was added to obtain a final concentration of 200 mg/L. Beads were prepared using the ionotropic gelation method using the jet technique. In this technique the mixture was added dropwise in a solution of 500 mM CaCl₂ at 0°C. The beads were washed with ultrapure water twice and stored at 4°C. The beads were also prepared without GA₃ (Blandón et al., 2016).

3.4.14 Encapsulation efficiency

The encapsulation of GA₃ in beads was determined by the quantification of GA₃ in CaCl₂ solution, after the beads formation (Blandón et al., 2016).

3.4.15 Preparation of GA₃ chitosan nanoparticles (CS)

Chitosan nanoparticles were prepared by the gelation method described by Pereira et al. (2017b). A CS solution (0.2% pH 4.5) was prepared in a solution of 1% acetic acid under vigorous stirring. Then, GA₃ was added to obtain a final concentration of 200 mg/L. A solution of 0.1% TPP (pH 4.5) at 4°C was added slowly to the CS solution after the dissolution of GA₃. 6 mL of the TPP solution was added to 10 mL of CS solution with GA₃. The nanoparticles were also prepared without GA₃.

3.4.16 Preparation of GA₃ chitosan-kefiran nanoparticles (CS/KEF)

Chitosan and kefiran nanoparticles were prepared based on the gelation method described by Pereira et al. (2017b). A CS solution (0.2% pH 4.5) was prepared in a solution of 0.5% acetic acid under vigorous stirring. A KEF solution (0.2%) was prepared in hot water. Then, the same volumes of CS and KEF solutions were blended. GA₃ was added to obtain a final concentration of 200 mg/L. A solution of 0.05 % TPP (pH 4.5) at 4°C was added slowly to the CS/KEF solution after the dissolution of GA₃. 6 mL of the TPP solution was added for each 10 mL of CS/KEF solution. The nanoparticles were also prepared without GA₃.

3.4.17 Preparation of GA₃ alginate-chitosan nanoparticles (ALG/CS)

GA₃ nanoparticles were prepared based on the ionotropic pregelation method described by Pereira et al. (2017b). 2.5 mL of a CaCl₂ solution (0.30%) were added to 37.17 mL of ALG solution (0.10%, pH 4.9) under vigorous stirring during 60 min. GA₃ was added to obtain a final concentration of 200 mg/L. 8.75 mL of CS solution (0.10%, pH 4.6), which was prepared in aqueous solution of acetic acid

(0.50%), were added for 90 min. Nanoparticles were also prepared without GA₃ as control.

3.4.18 Preparation of GA₃ alginate-chitosan-kefiran nanoparticles (ALG/CS/KEF)

GA₃ nanoparticles were prepared based on the ionotropic pregelation method described by Pereira et al. (2017b). 2.5 mL of a 0.21% CaCl₂ solution were added to a 37.17 mL of a solution consisting of ALG (0.07%) and KEF (0.07%) under vigorous stirring, at pH4.9 during 60 min. GA₃ was added to obtain a final concentration of 200 mg/L. 8.75 mL of CS solution (0.07%, pH 4.6), which was prepared in an aqueous solution of acetic acid (0.50%), were added to the ALG:KEF during 90 min. Nanoparticles were also prepared without the GA₃ as control.

3.4.19 Scanning electron microscopy (SEM)

Beads and nanoparticles were freeze-dried before SEM observations. Samples were prepared by sputtering the sample surface with gold using a metalizer. After that, samples surfaces and morphologies were observed by a TESCAN VEGA 3 LMU.

3.4.20 Statistical analysis

Analytical data was submitted to ANOVA and Duncan's test. Statistical analyses were conducted with the support of the software Statistica Version 7.0 (Minneapolis, USA).

3.4.21 Release assays

ALG beads containing standard GA₃ were added to Schott flasks containing phosphate buffer solution 0.2 M (pH 7.4). Flasks were then incubated in a water bath orbital shaker (Ethiktechnology) at 30°C, 100 rpm. Aliquots were withdrawn after 2, 4, 6, 8 and 24 hours. In order to maintain the same volume, the flask was refilled with fresh media (Blandón et al., 2016; Shukla et al., 2010; Soni et al., 2010). The same was done with the ALG/KEF beads. GA₃ concentration was determined using Agilent

Technologies 1260 Infinity HPLC. The column was Eclipse XDB-C18 (150 x 4.6 mm; 5 μm) and the mobile phase was 70% acetonitrile and 30% water (containing 0.1% phosphoric acid), pumped at a flow rate of 0.8 mL min^{-1} . The detector wavelength was 210 nm.

3.4.22 Germination test

Germination tests were carried out with the use of beads (ALG and ALG/KEF) and nanoparticles (CS, CS/KEF, ALG/CS and ALG/CS/KEF), with and without GA_3 , to evaluate the germination rate of *Glycine max*, soybean. First, seeds were prepared with their immersion during 1 hour in solutions containing 25 mL of the beads or nanoparticles and 25 mL of phosphate buffer so as final concentration of 100 mg/L released GA_3 . The control (soy seeds) was immersed only in phosphate buffer. A solution of free GA_3 was also analyzed. The seeds were placed in seed trays filled with vermiculite.

In a second step, GA_3 microspheres and nanoparticles (with and without pre-immersion) were placed with the seeds in seed trays filled with vermiculite. Trays were maintained in a greenhouse under natural conditions of light and temperature. There were 20 seeds for each treatment. Only the phosphate buffer was added to the control (soy seeds). For both cases, seed germination was evaluated every day. After 7 days, plants were collected and the root length was measured (Pereira et al., 2017a; Pereira et al., 2017b).

The germination percentage (%) is calculated by the equation below:

$$\text{Germination percentage (\%)} = \frac{\text{Number of germinated seeds}}{\text{Number of total seeds}} \times 100$$

The mean germination time is calculated by the equation below:

$$\bar{t} = \frac{\sum_{i=1}^k n_i t_i}{\sum_{i=1}^k n_i}$$

\bar{t} : mean germination time;

n_i : number of seeds germinated in the time i , not the accumulated number;

t_i : days from the beginning of the experiment to the i^{th} day;

k : last day of germination.

3.5 RESULTS AND DISCUSSION

3.5.1 Optimization of inoculum production

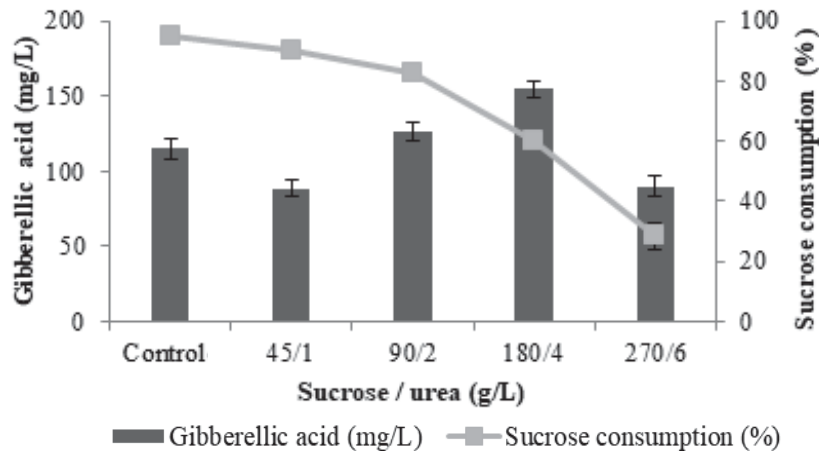
The ideal sucrose and urea concentrations to be added to CP/SHAE medium were determined with the support of the CCRD experimental design. CCRD results and ANOVA analysis are presented in the APPENDIX. The best biomass production (17.55 g/L) was obtained when 180 g/L of sucrose and 4 g/L of urea were added to the CP/SHAE medium. The ANOVA results showed a R^2 of almost 0.70, which is acceptable for bioprocesses. This condition promoted a 10-fold higher biomass concentration than the initial biomass production (1.66 g/L) without supplementary addition of sucrose and urea (data not shown). GA_3 production depends on a certain biomass formation, which certainly allows high yields of the secondary metabolite synthesis (Rodrigues et al., 2012).

3.5.2 GA_3 production by SSSF

GA_3 production was carried out by SSSF using CP/SH medium in suspension added of four different concentrations of sucrose (from 0 to 270 g/L) and urea (from 0 to 6 g/L). The results of GA_3 production and reducing sugars consumption are presented in FIGURE 7.

The best production of GA_3 by *G. fujikuroi* NRRL 2278 (154.44 mg/L) was obtained with the addition of 180 g/L of sucrose and 4 g/L of urea to CP/SH fermentation medium, after 5 days, which represents a productivity of 1.287 mg/L.h. Reducing sugars consumption was also high (60.24%), which indicates that the major part of the supplementary sucrose was consumed during the process. Páramo et al. (2004) reported a GA_3 production of 120 mg/L by *G. fujikuroi* NRRL 2278 using 25 g/L of glucose. Gökdere and Ateş (2014) obtained only 57.9 mg/L of GA_3 by submerged fermentation using also *G. fujikuroi* NRRL 2278, and a medium containing rice flour and 40 g/L of glucose.

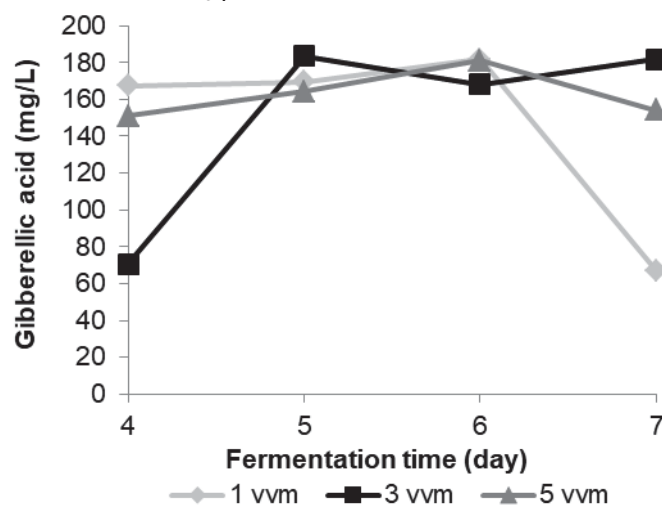
FIGURE 7. GA₃ production by SSSF using CP/SH medium with the addition of different concentrations of sucrose and urea.



3.5.3 Scale-up of GA₃ production in BCR

Kinetics of GA₃ production *G. fujikuroi* NRRL 2278 was studied using BCR and STR type bioreactors. The goal of this study was to determine the best conditions of aeration and agitation for the process. In BCR, fermentation essays were performed using the optimized medium composition determined in flasks. The influence of different aeration rates (1, 3 and 5 vvm) on GA₃ production was studied (FIGURE 8).

FIGURE 8. Kinetics of GA₃ production with different aeration rates in BCR.



Samples were withdrawn each day, from the 4th day, when GA₃ production begins. The aeration rate of 3 vvm promoted the best GA₃ production (183.31 mg/L)

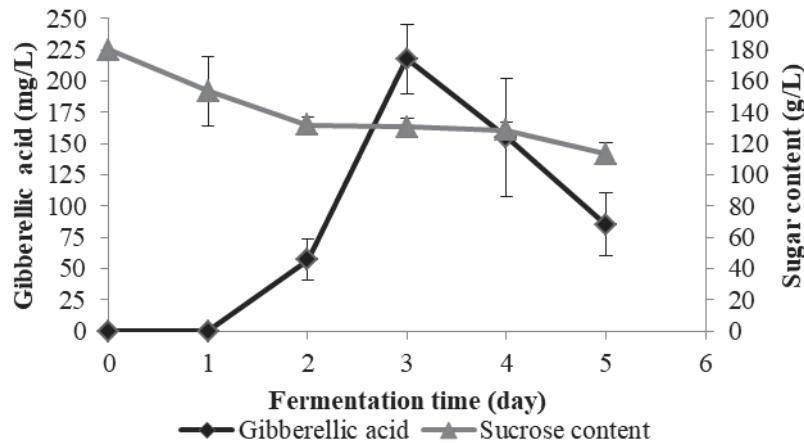
on the 5th day of fermentation reaching a productivity of 1.53 mg/L.h. A gain of 18.69% was obtained in BCR comparing with GA₃ production that was reached in flasks (151.44 mg/L). Hence, the forced aeration has a positive influence on GA₃ synthesis, leading to great perspectives for process scale-up.

According to Oliveira (2012), GA₃ concentration reached 208 mg/L after 9 days in BCR, representing a productivity of 0.96 mg/L.h, which was 38% lower than the productivity obtained in this work (1.53 mg/L.h). No other reports were found about GA₃ production in BCR.

3.5.4 Kinetics of GA₃ production in STR

GA₃ production in STR was performed using the optimized medium composition such as it was employed in flasks. The aeration rate was 3 vvm, due to the result obtained in BCR. The agitation rate was 500 rpm, according to Oliveira (2012). Samples were withdrawn each 24 hour for GA₃ analysis of and sugars consumption. The results obtained are presented in FIGURE 9. GA₃ production started after the 1st day and reached a higher production on the 3rd day (217.67 mg/L), after that, the production decreased. GA₃ production was carried out using different fermentation systems, each one with this own characteristics of aeration and agitation, which certainly influenced the process. GA₃ production reached: in flasks (154.44 mg/L), in BCR (183.31 mg/L) and in STR (217.67 mg/L). With these results, it is possible to affirm that STR was favorable to GA₃ production, probably due to the higher agitation promoted that increased the medium homogenization and oxygen transfer, which improved biomass and GA₃ production.

The researches described by Oliveira (2012) and Páramo et al. (2004) in SmF, indicate that the results reported in the present work are significant and promising. Oliveira (2012) obtained a GA₃ productivity of 2.84 mg/L.h in STR by a strain of *G. fujikuroi* using a similar medium with CP. Páramo et al. (2004) reached a GA₃ production of 206 mg/L performed in STR during 15 days with the higher production on the 14th day, but, productivity was only 0.61 mg/L.h.

FIGURE 9. Kinetics of GA₃ production and sugar consumption in STR using CP/SH medium.

3.5.5 Encapsulation of GA₃

3.5.5.1 GA₃ beads

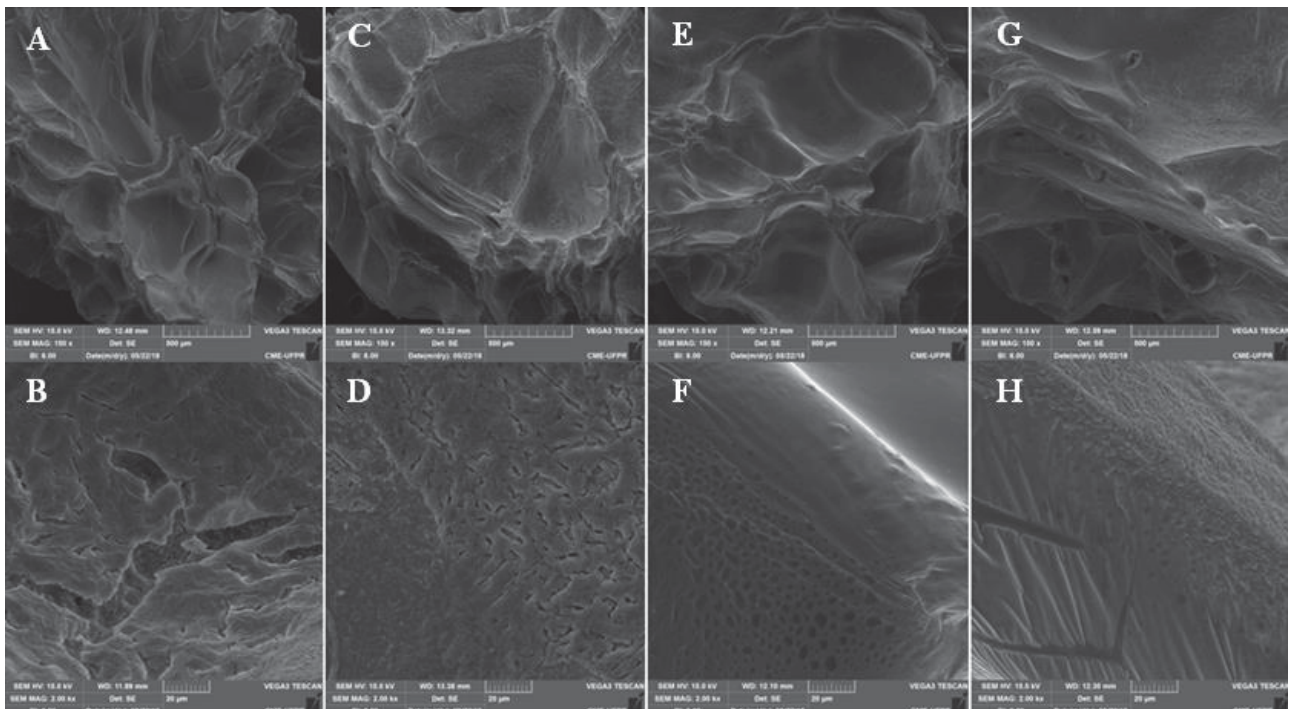
ALG and ALG/KEF beads were prepared by ionotropic gelation method. They presented a spherical shape in aqueous solution even when they are loaded with GA₃. Beads' mean diameter was about 4 mm for ALG, and 3.5 mm for ALG/KEF with or without GA₃. It was not possible to produce beads of pure kefiran by the same method, probably due to its gelation properties.

The encapsulation efficiency of GA₃ was about 70% and 60%, for ALG beads and ALG/KEF beads, respectively. Segale et al. (2016) described the encapsulation of Celecoxib by alginate beads with encapsulation efficiency about 40%, which was lower than the one obtained in this work. However, the encapsulation efficiency obtained in this work for ALG/KEF beads was lower than 80% obtained by Blandón et al. (2016) for ciprofloxacin encapsulation. The hypotheses to explain that is the nature of encapsulated biomolecule and biopolymer, and the chemical interactions between them.

The polymerization reaction occurs when the alginate, that has negative charge, is added to CaCl₂ solution and the carboxylic acid groups interact with the Ca²⁺ ions forming an "egg-box" structure. The carboxylic acid groups of GA₃ contribute to its incorporation in the polymeric mesh formed, because of the same chemical interactions (Azevedo et al., 2014; Pereira et al., 2017b). ALG/KEF beads have a lower encapsulation efficiency compared to the ALG beads probably because of the addition of kefiran that competes with GA₃ for alginate binding. So, the

interactions between the polymers and GA₃ decrease and an inefficient matrix was formed. FIGURE 10 represents the images obtained by SEM for ALG, ALG/KEF, ALG-GA₃ and ALG/KEF-GA₃ beads. An irregular surface of the beads can be observed in FIGURE 10A, C, E and G. Beads were freeze-dried for SEM analysis and, because of that, ALG and ALG/KEF beads, with and without GA₃, showed a shrinkable matrix causing the loss of spherical morphology.

FIGURE 10. SEM images of beads: ALG at 250 x (A) and 2 kx (B); ALG-GA₃ at 250 x (C) and 2 kx (D); ALG/KEF at 250 x (E) and 2 kx (F); and ALG/KEF-GA₃ at 250 x (G) and 2 kx (H).



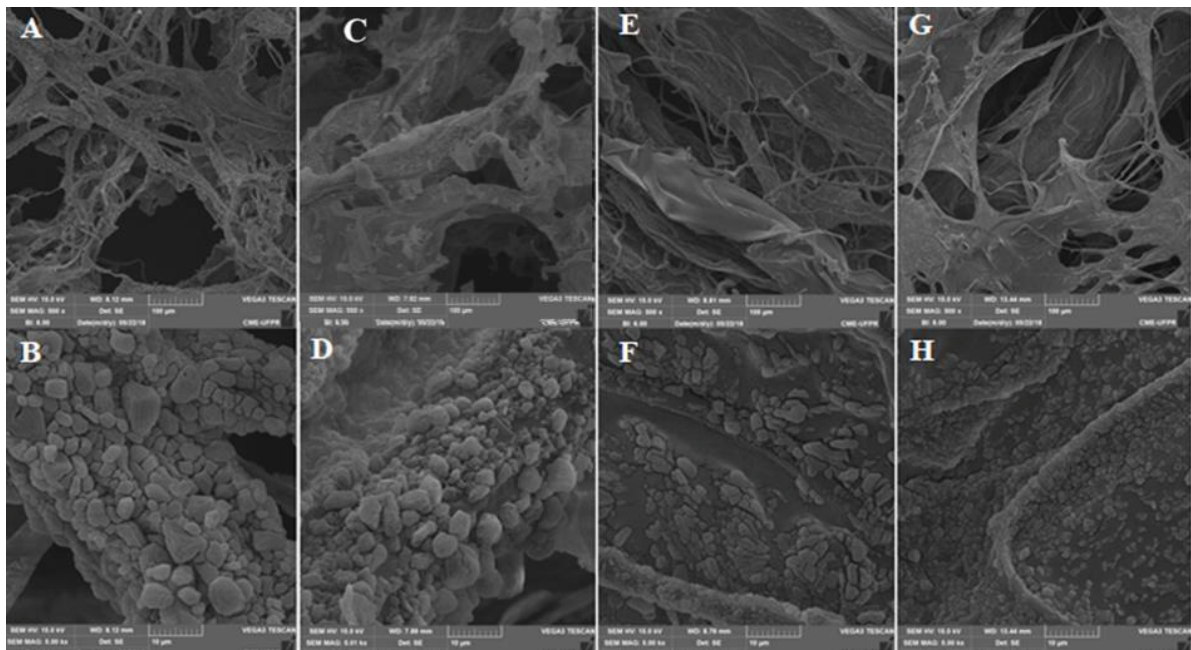
The addition of KEF in the formulation promoted the formation of beads with a smoother surface when compared to ALG and ALG-GA₃ beads. This was also observed by Blandón et al. (2016). The ALG/KEF and ALG/KEF-GA₃ beads exhibited porous surfaces with only few cracks in the structures. As far as we know, there are no reports about GA₃ encapsulation in ALG or ALG/KEF beads. So, it is the first work using this system for a PGR encapsulation.

3.5.5.2 GA₃ nanoparticles

Images of CS, CS-GA₃, CS/KEF and CS/KEF-GA₃ nanoparticles were analysed by SEM and are presented in FIGURE 11. The formation of nanoparticles

only occurred for the formulation of chitosan with and without GA₃ (FIGURE 11 B and D). As it can be observed, the size of the particles varies with nanoparticles, which have almost 850 -950 nm, and microparticles that can reach 10 µm. Pereira et al. (2017b) obtained nanoparticles of CS loaded with GA₃ with an average of 270 nm. The size of the nanoparticles varied with pH and temperature. In the study of Rampino et al. (2013), the aspects of CS nanoparticles after drying was similar to the nanoparticles produced in this work. According to these authors, after lyophilisation or spray-drying, nanoparticles form aggregates, which increases in size.

FIGURE 11. SEM images of nanoparticles: CS at 500 x (A) and 5 kx (B); CS-GA₃ at 500 x (C) and 5 kx (D); CS/KEF at 500 x (E) and 5 kx (F); and CS/KEF-GA₃ at 500 x (G) and 5 kx (H).



It is possible to see that nanoparticles are adhered to fibers. Contrarily, CS/KEF nanoparticles, with and without GA₃ formulations, did not form nanoparticles, where it is possible to verify a certain phase segregation (FIGURE 11 F and H). The reasons that could cause this phenomenon are the concentration of each polymer, the low degree of homogenization of the solution, and the low interaction between the matrix and GA₃. FIGURE 11 E and G showed a kind of film that was formed by KEF, with a smooth surface.

ALG/CS, ALG/CS-GA₃, ALG/CS/KEF and ALG/CS/KEF-GA₃ formulations were not appropriate for producing nanoparticles. A type of film was formed in these cases and it is possible to observe the presence of some precipitates. These

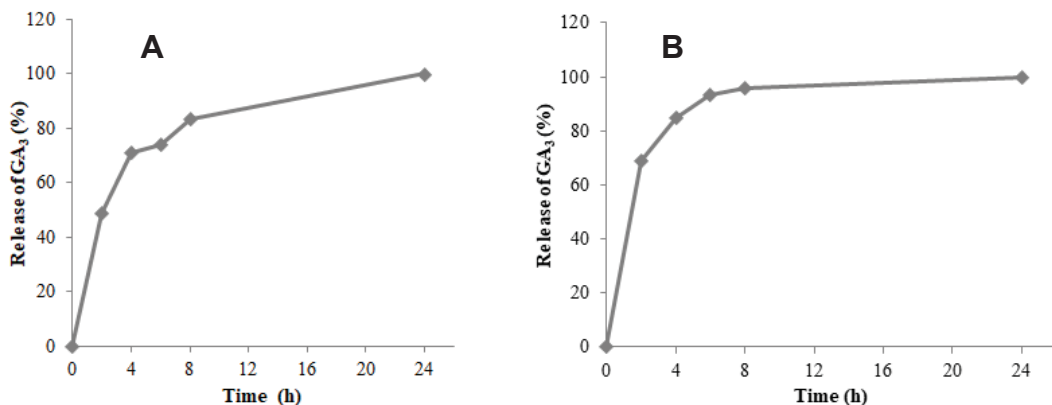
precipitates could be caused by the reaction of the polysaccharides with the CaCl_2 used in the formulation, or the GA_3 in the case of ALG/CS- GA_3 . The problems with precipitation and nanoparticles formation could be related to the inadequate concentration of each polysaccharide, low degree of homogenization of the solution, and the method of preparation. In the ALG/CS/KEF- GA_3 system there were no precipitates. KEF formulations led to the formation of films. As it was observed for the nanoparticles CS/KEF and CS/KEF- GA_3 , Kefiran, promotes again de formation of with a smooth surface, which may bring good perspectives for GA_3 stability and controlled release for different applications.

The aspect of the film formed for the ALG/CS formulation were similar to the ALG/CS film formed by Conzatti et al. (2017), after the process of freeze-drying. Karki et al. (2016) described the study of thin films as a new drug delivery tool, where ALG and CS were employed.

3.5.6 Release assays

After the study of GA_3 beads or nanoparticles encapsulation, release assays were carried out to evaluate the percentage of release with time. GA_3 release curves were determined using ALG- GA_3 and ALG/KEF- GA_3 beads submerged in the phosphate buffer solution pH 7.4. The release curves were constructed considering that, after 24 hours from the beginning of the experiment, the concentration of released GA_3 in the solution was 100% (FIGURE 12).

FIGURE 12. Release profiles of GA_3 encapsulated in ALG (A) and ALG/KEF (B) beads.



GA₃ release rate of the ALG-GA₃ beads was slower than the observed for the ALG/KEF-GA₃ system. For the first 2 hours, the ALG-GA₃ system released about 48% of GA₃ and the ALG/KEF-GA₃ released about 68%. In both cases the release curves showed the pattern expected for release assays. ALG/KEF beads reached a little bit higher GA₃ release percentage in the same time than only ALG beads. In this case, it can be suggested that the matrix formed by alginate and kefir was not stable, which allowed the release of the PGR. This result was expected since, from encapsulation efficiency, it was observed that the addition of kefir promotes an inefficient matrix, due to the competition with GA₃ for alginate binding. So, the chemical interaction is affected, causing an effect in the release rate (Azevedo et al., 2014; Pereira et al., 2017b). The release curves obtained in this work suggested that the ALG was able to produce a more stable gel network with GA₃, resulting in a slower release.

Blandón et al. (2016) described a different behavior of the ALG and ALG/KEF microspheres for the controlled release of ciprofloxacin. Differences in the release curves could be related to the interaction of each biomolecule with ALG or ALG/KEF matrix, the porosity, the size of the molecules, and the composition and degree of purification of KEF. The nanoparticles systems were not tested in the present study in terms of controlled release, because it was not possible to obtain stable CS/KEF, ALG/CS, and ALG/CS/KEF nanoparticles. Further studies will be conducted to test CS microparticles controlled release.

3.5.7 Germination assays

Germination assays were carried out with the immersion of seeds in suspensions containing different beads and nanoparticles GA₃ systems as it is shown in TABLE 6. ALG-GA₃ system was the only to promote 100% of germination (TABLE 6). The treatments: ALG-GA₃, ALG/KEF-GA₃ and CS-GA₃ showed a higher germination percentage than the systems without GA₃, which proves that GA₃ was able to promote seed germination.

TABLE 6. The germination percentage, mean germination time, and root length of soybean for each treatment for the first germination assay.

Treatment	Germination percentage	Mean germination time (day)	Root length (cm)
Control	40%	6.375	3.2 ± 1.1 ade
GA ₃ free	65%	6.154	3.1 ± 0.7 ade
ALG	61%	5.545	3.1 ± 0.5 ade
ALG-GA ₃	100%	5.800	3.2 ± 0.7 ade
ALG-KEF	70%	5.928	2.7 ± 0.7 abcde
ALG-KEF-GA ₃	85%	6.176	3.0 ± 0.7 acde
CS	60%	5.667	2.2 ± 0.7 bcd
CS-GA ₃	75%	5.933	2.3 ± 0.6 bcd
CS-KEF	65%	6.077	2.4 ± 0.9 bcde
CS-KEF-GA ₃	65%	5.308	2.3 ± 0.5 bcd
ALG-CS	70%	5.428	2.4 ± 0.6 bcde
ALG-CS-GA ₃	55%	6.182	2.2 ± 0.7 bcd
ALG-CS-KEF	65%	6.154	2.6 ± 0.4 abcde
ALG-CS-KEF-GA ₃	60%	6.000	2.7 ± 0.7 abcde

Means the same letter for each parameter are not different at $p < 0.05$ by Duncan's test

In the case of ALG/CS, ALG/CS-GA₃, ALG/CS/KEF and ALG/CS/KEF-GA₃ treatments the difference was not the expected showing that the germination percentage did not increase with the addition of GA₃. This could be related to the formulation method of these nanoparticles, and the CaCl₂ content. The exogenous application of CaCl₂ is may also promote seed germination, and increased endogenous levels of GA₃ and indole-acetic acid, could be one of the reasons why these systems with and without GA₃ did not present significant difference (Pereira et al., 2017b; Wang et al., 2016). All treatments presented higher germination percentages than the control. According to França-Neto et al. (2016) and Morais et al. (2016), the harvesting and processing of seeds led to lost in seeds' quality, affecting the seeds' germination. The low germination percentage causes financial loss to the farmers, increasing the price of soybean.

The shortest mean germination time was obtained for CS/KEF-GA₃ treatment; this could be related to the polymers properties, GA₃ concentration released in the buffer solution, and soybean seeds' absorption of GA₃ in this treatment. All treatments had shorter mean germination time than the control. Thus, the studied GA₃ beads and nanoparticles accelerate the germination, to break seeds' dormancy.

The root length of soybean seeds treated by CS, CS-GA₃, CS/KEF, CS/KEF-GA₃, ALG/CS, and ALG/CS-GA₃ systems were statistically different to the root length of the control. The root length was shorter in these treatments than in the control. This could be related to the short immersion time (1 hour), so the concentration of the available GA₃ was low. Another explanation for that could be the pH of the nanoparticles' solutions, the acid pH (4-4.5) could have induced the lower growth rate of the roots (Foy, 1992). The other systems did not show statistical difference with the control, showing that GA₃ had no positive effect on root growth. This could be also related to the fact that all the seeds were immersed into phosphate buffer solution, increasing the K and P uptake, which could lead to an increase in root length (Assefa, 2008).

The results obtained in the second germination tests, which consisted in the addition of beads and nanoparticles in vermiculite, are presented in TABLE 7.

TABLE 7. The germination percentage, mean germination time, and root length of soybean for each treatment for the second germination assay.

Treatment	Germination percentage	Mean germination time (day)	Root lenght (cm)
Control	85%	3.882	2.5 ± 0.5 abdegh
GA ₃ free	95%	3.842	2.4 ± 1.0 abdegh
ALG	100%	3.750	3.0 ± 0.8 cefh
ALG-GA ₃	80%	4.188	2.9 ± 0.8 acefh
ALG-KEF	90%	3.833	2.7 ± 0.9 abcegh
ALG-KEF-GA ₃	95%	3.895	3.3 ± 1.3 cfh
CS	95%	4.158	2.2 ± 0.4 abdeg
CS-GA ₃	100%	3.900	1.9 ± 0.6 abdg
CS-KEF	95%	3.737	3.0 ± 0.7 acefh
CS-KEF-GA ₃	100%	3.700	2.3 ± 0.8 abdeg
ALG-CS	95%	3.474	2.2 ± 0.6 abdeg
ALG-CS-GA ₃	100%	3.750	2.2 ± 0.5 abdeg
ALG-CS-KEF	100%	3.650	2.0 ± 0.4 abdg
ALG-CS-KEF-GA ₃	90%	3.333	2.0 ± 0.7 abdg

Means the same letter for each parameter are not different at p<0.05 by Duncan's test

In this case, germination percentages were higher than in the first experiment, this could be related to the increase of temperature and luminosity. ALG, CS-GA₃, CS/KEF-GA₃, ALG/CS-GA₃ and ALG/CS/KEF treatments presented 100% of germination. For ALG and ALG/CS/KEF systems, CaCl₂, which is a part of its

formulation, could be the responsible for germination (Wang et al., 2016). CS-GA₃, CS/KEF-GA₃, ALG/CS-GA₃ systems had the influence of GA₃ in the germination.

ALG/CS/KEG-GA₃ nanoparticle system showed the shortest mean germination time. This system contains GA₃, which may be responsible to decrease the mean germination time. The other treatments did not have significant influence on germination time comparing to the control. The fact that some treatments showed higher mean germination time than the control could be related to the low GA₃ release, the formulation, and the pH.

Comparing the root length, only two treatments, ALG and ALG/KEG-GA₃, are statistically different from the control. They presented higher root growth (3.0 and 3.3 cm, respectively) than the control (2.5 cm). Plant roots are responsible for nutrients and water uptake from the soil, so, their development is very important (Pereira et al., 2017b). Highest root length was observed for the ALG/KEF-GA₃ treatment, showing that GA₃ really influenced the growth as it was released. This could be economically important for agriculture countries, where the gain in productivity of different cultivars is imperative. The other treatments did not show a statistical difference compared to the control.

Thus, in the case of beads, it is more interesting, in terms of root length, to put them directly in the soil instead of promoting the immersion of the seeds, due to the controlled release. The free GA₃ worse performance, in both experiments, could be explained by its degradation due to light and water, and the different interactions, due to pH and soil composition. In the case of both beads and nanoparticles, GA₃ was protected by two matrix systems, preserving its properties (Machado and Soccol, 2008; Preez et al., 1993).

3.6 CONCLUSIONS AND PERSPECTIVES

GA₃, an important plant growth hormone, was produced using simple and economic conditions with the re-use of citric pulp (CP) and soybean husks (SH), which are highly generated in different agriculture countries, such as Brazil. Optimal conditions were established in Erlenmeyer flasks reaching 154.44 mg/L, using a medium composed of 3.5 % (w/v) CP, 1.5 % (w/v) SH, 180 g/L sucrose and 4 g/L urea. The process was also conducted in different models of bioreactors, BCR and STR, where GA₃ production reached 183.32 mg/L and 217 mg/L, in BCR and STR,

respectively. This could be related to the better aeration and agitation promoted by these types of bioreactors. The formation of beads and nanoparticles loaded with GA₃ allowed the study of beads and nanoparticles systems for GA₃ controlled release.

Germination assays showed that the beads and nanoparticles, with and without GA₃, impacted on seeds' germination and root length. For seeds' germination the ALG-GA₃ system was the best, promoting 100% of germination, when seeds were previously immersed in the solution, contrary to the control that obtained led to only 40%. In the terms of root length the ALG/KEF-GA₃ treatment promoted a higher length of the roots when added to the vermiculite. New assays must be performed to improve the formation of nanoparticles loaded with GA₃. For that, studies of polysaccharides' concentration, agitation, pH and other factors could be evaluated. Besides, other cultivars and field assays should also be conducted.

4 GENERAL CONCLUSION

The gibberellic acid is a high-value plant growth regulator that is used in different crops to improve the growth and development of plants. In chapter 1, the bibliographic review describes several techniques used in GA₃'s production, formulation, and encapsulation to guarantee the controlled delivery of the biomolecule. Based on the bibliographic review, the production of GA₃ occurs mainly by submerged fermentation using *Gibberella fujikuroi*, and formulation is a relevant step to the biomolecule stabilization. Currently, the commercial formulations available are liquid and solid. However, these formulations can present some disadvantages as to be toxic to the environment and to dispose of dust.

Encapsulation is an important alternative to overcome these formulations disadvantages. The application of nanotechnology allows the production of nanoparticles loaded with biomolecules, which can be delivered in a controlled way leading to higher stability of it. A few papers about controlled delivery of GA₃ are described; the vast majority is related to the use of chitosan in the systems. Based on that, there is an opportunity to explore controlled delivery systems of GA₃ using different biopolymers, looking for better activity and higher stability of the regulator in crops.

Chapter 2 relates the experiments done based on the information obtained in chapter 1. An alternative to reduce the production costs of GA₃ was the use of agro-industrial wastes, citric pulp and soybean husk, as a substrate in semisolid fermentation. The optimized conditions of GA₃ production in flasks allowed to reach 154,44 mg/L of GA₃, medium composition: 3.5% (m/v) citric pulp; 1.5% (m/v) soybean husk; 180 g/L of sucrose and 4 g/L of urea. To study the scale-up of the process, GA₃ was also produced in bubble column reactor and STR. In these cases, the GA₃ concentrations obtained were 183.32 mg/L and 217 mg/L, respectively. The concentrations obtained in the bioreactors were higher than the one observed in flasks and, this can be related to the better agitation and aeration promoted by the bioreactors systems. Hence, this represents a great perspective to scale-up the process of plant growth regulator production using STR. Other alternatives to reach higher yields would be the study of genetically modified microorganisms, different fermentation techniques, and optimization of the process conditions in a bioreactor.

The study of GA₃ controlled delivery was carried out after the production of beads and microparticles. These systems were loaded with the regulator, using different biopolymers blended or not and applied in soybean cultivar. The germination tests showed that the beads and microparticles, with and without GA₃, affected the seeds' germination and the root length. All seeds germinated when were treated previously with alginate and GA₃ system, comparing with the control that was about 40% of germination. This fact is relevant due to the farmers' financial loss caused by the low germination percentage that is directly related to the increase in the soybean price.

The seeds treated with the presence of beads system formulated by the combination of alginate, kefir and GA₃ in vermiculite showed higher root length. These results would cause a positivity impact in agro-industrial countries, focusing on the gain of productivity of different cultivars. As a perspective, other studies should be conducted to improve and promote the production of nanoparticles loaded with GA₃. It is important to focus on different biopolymers, concentrations, agitation, pH, temperature, and other variables that could be related to improving the formulation and encapsulation.

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APPENDIX 1 – PRODUCTION AND OPTIMIZATION OF INOCULUM

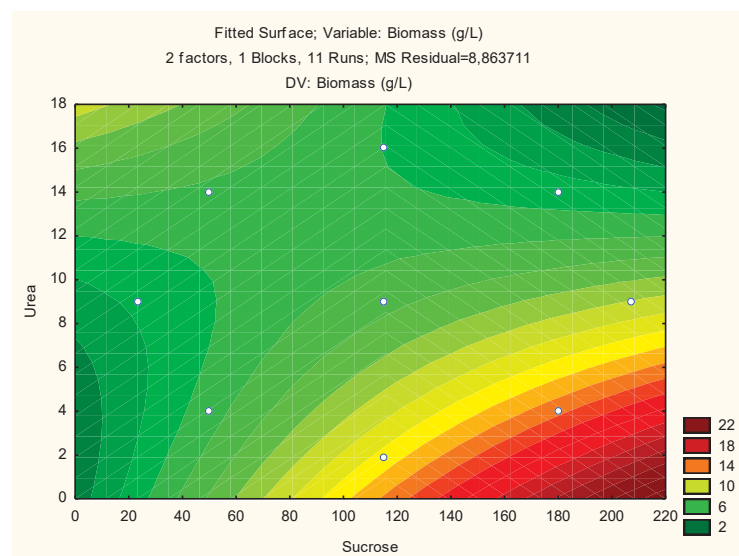
Biomass production in CP/SHAE medium with addition of different sucrose and urea concentrations.

Sucrose (g/L)	Urea (g/l)	Biomass (g/L)
50.00	4.00	7.12
50.00	14.00	5.75
180.00	4.00	17.55
180.00	14.00	6.66
23.08	9.00	3.92
206.92	9.00	5.55
115.00	1.93	8.33
115.00	16.07	4.25
115.00	9.00	6.76
115.00	9.00	6.08
115.00	9.00	7.17

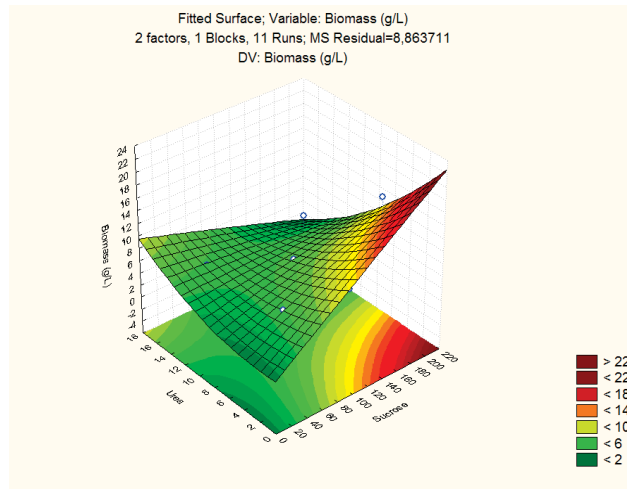
ANOVA table obtained as result of CDDR of the inoculum optimization.

ANOVA; Var.:Biomass (g/L); R-sqr=.67047; Adj.:.34094 (DCCR-Gabrielle-dados) 2 factors, 1 Blocks, 11 Runs; MS Residual=8,863711 DV: Biomass (g/L)					
Factor	SS	df	MS	F	p
(1)Sucrose (L)	23,2835	1	23,28348	2,626832	0,165997
Sucrose (Q)	0,0049	1	0,00487	0,000550	0,982201
(2)Urea (L)	40,6659	1	40,66586	4,587904	0,085108
Urea (Q)	3,1606	1	3,16061	0,356578	0,576423
1L by 2L	22,6766	1	22,67664	2,558369	0,170607
Error	44,3186	5	8,86371		
Total SS	134,4897	10			

Contour plot of CDDR of the inoculum optimization.

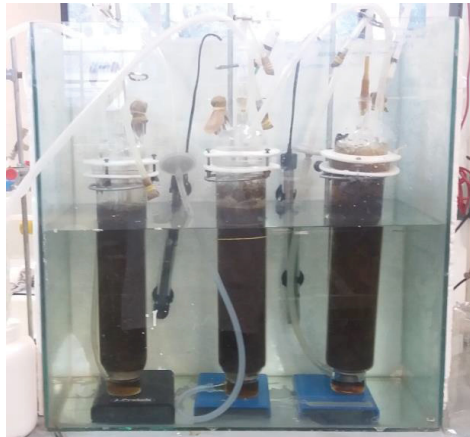


Surface plot of CDDR of the inoculum optimization.

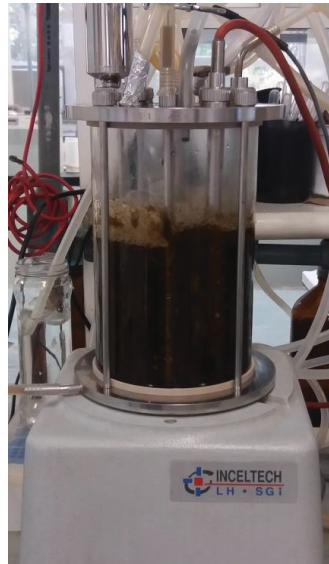


APPENDIX 2 – PRODUCTION AND PURIFICATION OF GIBBERELIC ACID

Production of GA₃ in BCR.



Production of GA₃ in STR.

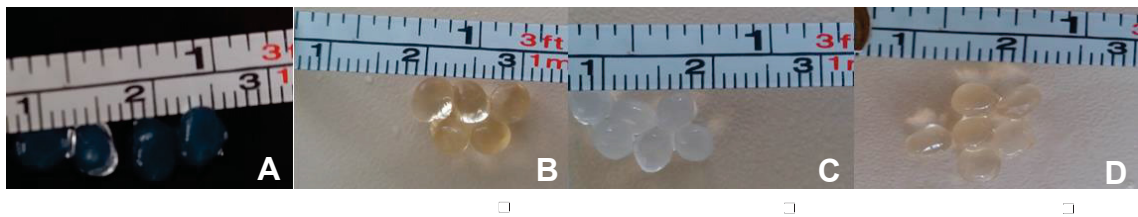


Membrane filtration system.

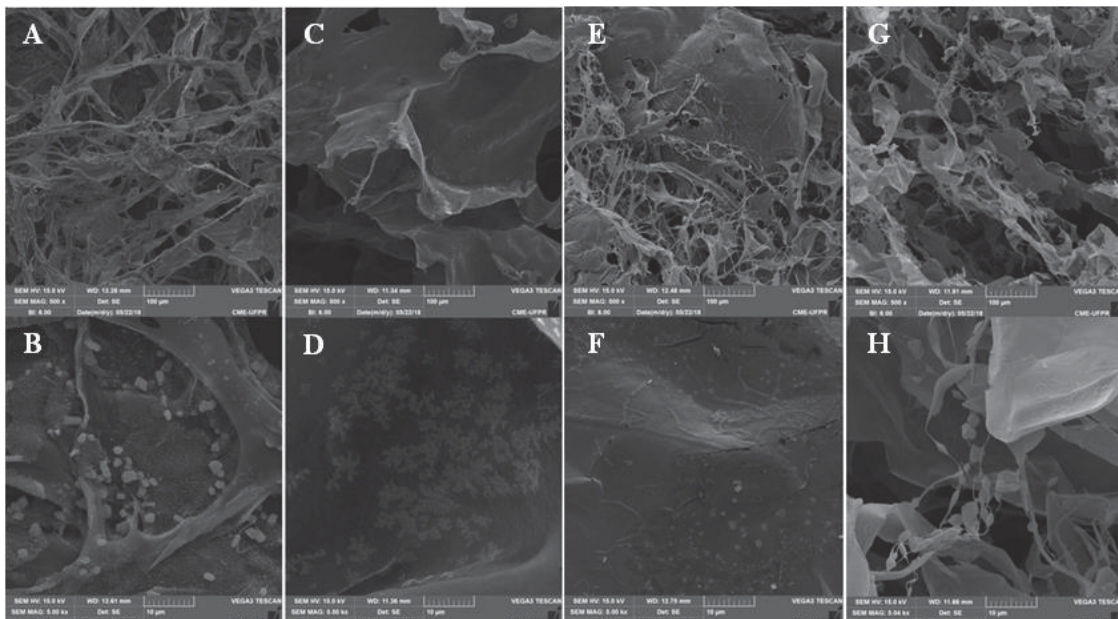


APPENDIX 3 – BEADS AND NANOPARTICLES, WITH AND WITHOUT GIBBERELIC ACID

ALG beads (A), ALG-GA₃ beads (extract) (B), ALG/KEF beads (C), and ALG/KEF-GA₃ beads(D).

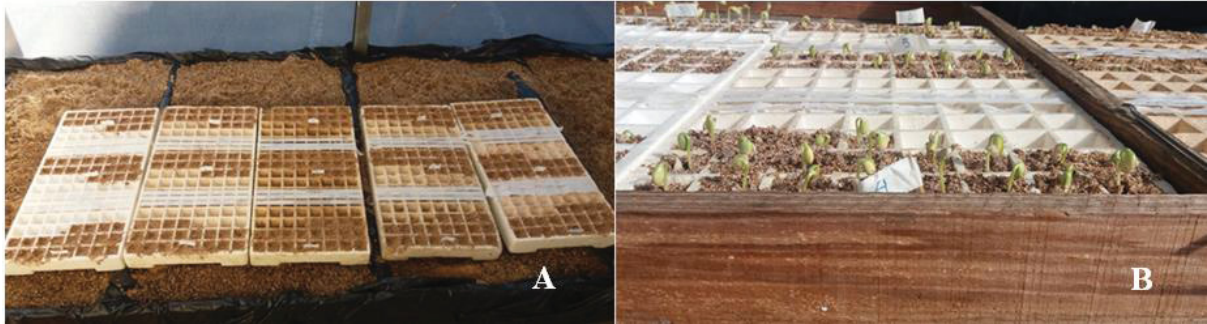


SEM images of nanoparticles: ALG/CS at 500 x (A) and 5 kx (B); ALG/CS-GA₃ at 500 x (C) and 5 kx (D); ALG/CS/KEF at 500 x (E) and 5 kx (F); and ALG/CS/KEF-GA₃ at 500 x (G) and 5 kx (H).

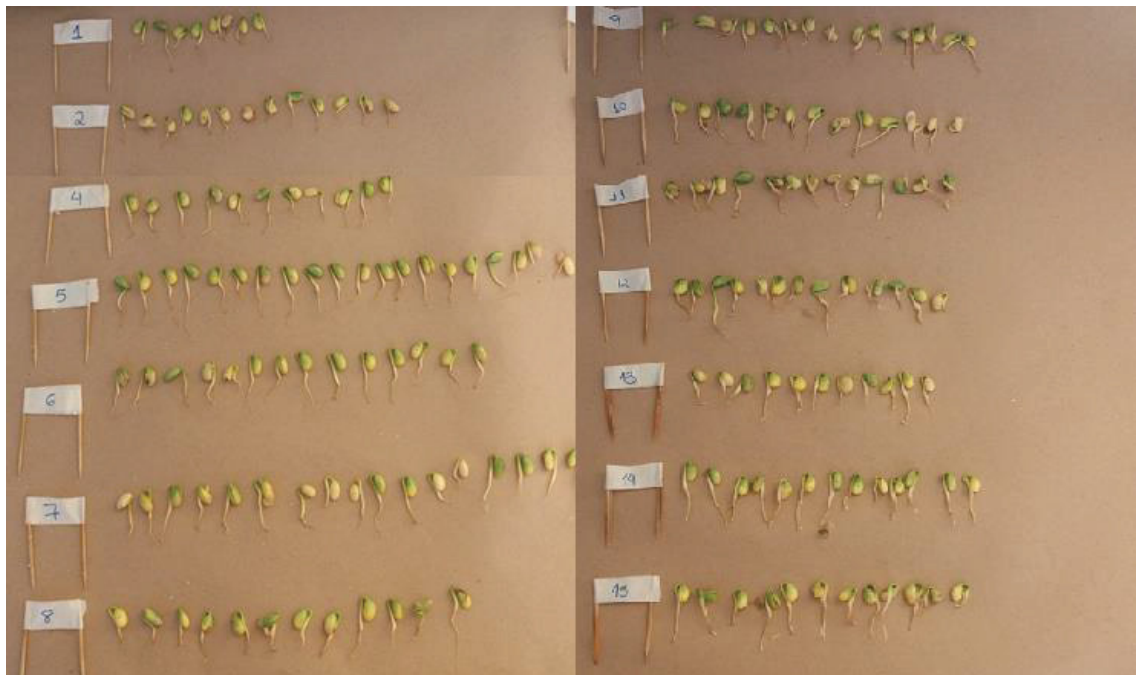


APPENDIX 4 – GERMINATION ASSAYS

Seed trays used in the germination assays (A) and sprouted seeds obtained in the second germination assay (B).



The soybeans germinated in all treatments tested in the first germination assay. 1 – Control; 2 – free GA_3 extract; 4 – ALG; 5 – ALG- GA_3 ; 6 – ALG/KEF; 7 – ALG/KEF- GA_3 ; 8 – CS; 9 – CS- GA_3 ; 10 – CS/KEF; 11 – CS/KEF- GA_3 ; 12 – ALG/CS; 13- ALG/CS- GA_3 ; 14 – ALG/CS/KEF; 15 – ALG/CS/KEF- GA_3 .



The soybeans germinated in all treatments tested in the second germination assay. 1 – Control; 2 – free GA₃ extract; 4 – ALG; 5 – ALG-GA₃; 6 – ALG/KEF; 7 – ALG/KEF-GA₃; 8 – CS; 9 – CS-GA₃; 10 – CS/KEF; 11 – CS/KEF-GA₃; 12 – ALG/CS; 13- ALG/CS-GA₃; 14 – ALG/CS/KEF; 15 – ALG/CS/KEF-GA₃.

