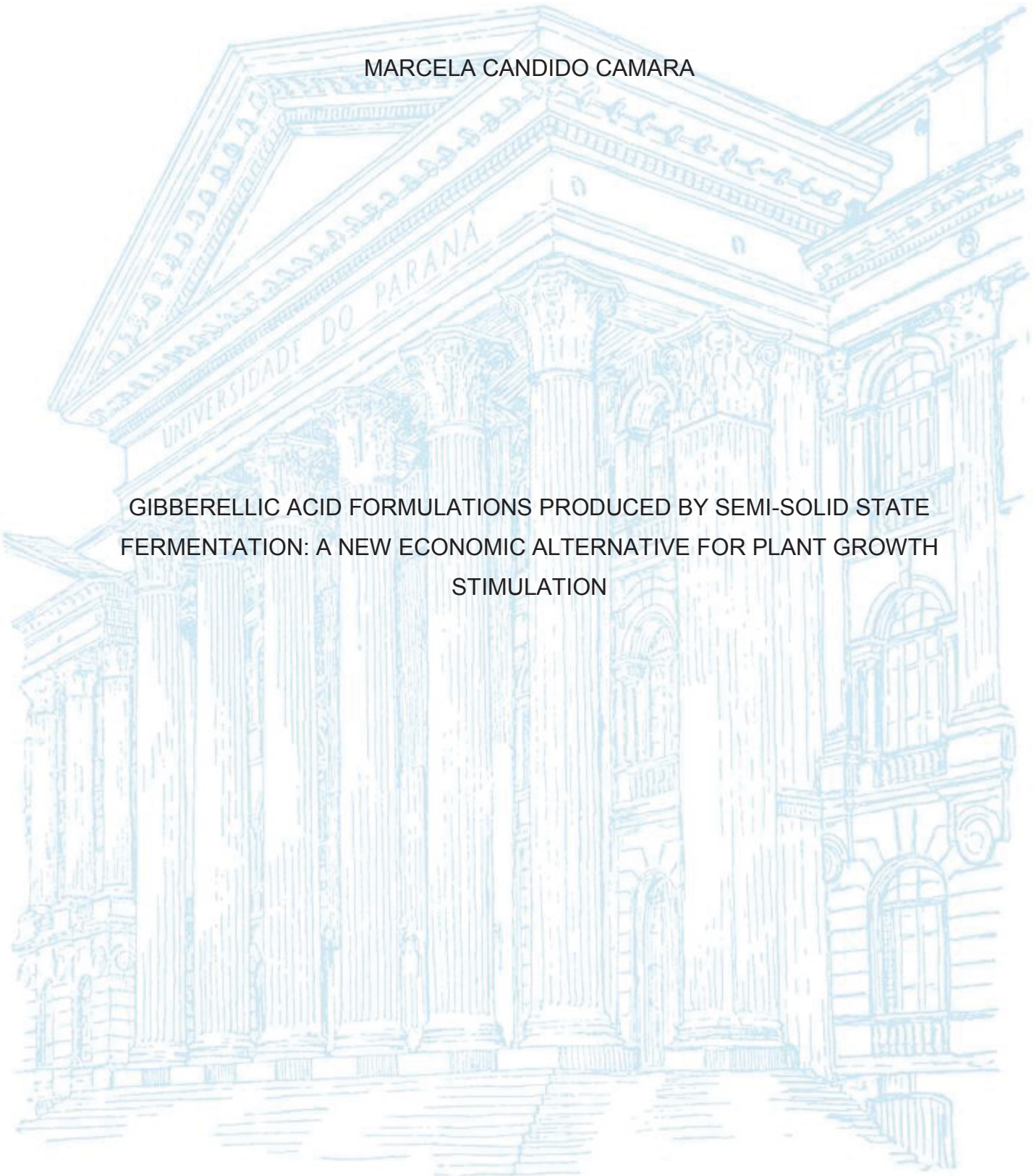
UNIVERSIDADE FEDERAL DO PARANÁ

MARCELA CANDIDO CAMARA



GIBBERELLIC ACID FORMULATIONS PRODUCED BY SEMI-SOLID STATE FERMENTATION: A NEW ECONOMIC ALTERNATIVE FOR PLANT GROWTH STIMULATION

CURITIBA

2018

MARCELA CANDIDO CAMARA

GIBBERELLIC ACID FORMULATIONS PRODUCED BY SEMI-SOLID STATE FERMENTATION: A NEW ECONOMIC ALTERNATIVE FOR PLANT GROWTH STIMULATION

Tese apresentada como requisito parcial à obtenção do grau de Doutor em Engenharia de Bioprocessos e Biotecnologia, no Curso de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, da Universidade Federal do Paraná.

Orientadora: Profª. Drª. Luciana Porto de Souza Vandenberghe
Co-orientador: Profº. Dr. Carlos Ricardo Soccol

CURITIBA

2018

Catalogação na Fonte: Sistema de Bibliotecas, UFPR
Biblioteca de Ciência e Tecnologia

C172g

Camara, Marcela Candido
Gibberellic acid formulations produced by semi-solid state fermentation: a new economic alternative for plant growth stimulation / Marcela Candido Camara. – Curitiba, 2018.

Tese - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, 2018.

Orientador: Luciana Porto de Souza Vandenberghe – Coorientador: Carlos Ricardo Soccol.

1. Ácido giberélico. 2. Fusarium. 3. Polpa seca de cítricos. 4. Soja – Biotecnologia. 5. Crescimento (Plantas). I. Universidade Federal do Paraná. II. Vandenberghe, Luciana Porto de Souza. III. Soccol, Carlos Ricardo. IV. Título.

CDD: 546.24

Bibliotecário: Elias Barbosa da Silva CRB-9/1894

MINISTÉRIO DA EDUCAÇÃO
SETOR TECNOLOGIA
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA

TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **MARCELA CÂNDIDO CÂMARA** intitulada: **GIBBERELLIC ACID FORMULATIONS PRODUCED BY SEMI-SOLID STATE FERMENTATION: A NEW ECONOMIC ALTERNATIVE FOR PLANT GROWTH STIMULATION**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 06 de Julho de 2018.

LUCIANA PORTO DE SOUZA VANDENBERGHE
Presidente da Banca Examinadora

CARLOS RICARDO SOCCOL
Avaliador Interno

JOÃO CARLOS BESPALHOK FILHO
Avaliador Externo

CRISTINE RODRIGUES
Avaliador Externo

ORANYS ISAAC MARIN GONZALEZ
Avaliador Externo

Dedico este trabalho aos meu pais,
por sempre me apoiarem e me
incentivarem nas minhas escolhas.

AGRADECIMENTOS

À minha família por confiarem em mim, pelo apoio e pela oportunidade de continuar nos estudos.

À minha orientadora, Profª. Drª. Luciana Porto de Souza Vandenberghe pela confiança em meu trabalho, pelas oportunidades que me foram concedidas e pela dedicação em me orientar, a qual foi fundamental para a conclusão deste trabalho.

Aos meus queridos amigos do laboratório, pela amizade, pela ajuda, por terem me dado força nos momentos difíceis e pelos momentos de boas risadas.

Aos professores do departamento de Engenharia de Bioprocessos e Biotecnologia, em especial o Profº. Dr. Carlos Ricardo Soccol, pela confiança e oportunidade de desenvolver esse trabalho.

Aos professores da banca examinadora, pela disponibilidade em avaliar e contribuir para a qualidade desta tese.

Ao Profº. Dr. João Carlos Bespalhok Filho por gentilmente ceder um espaço na casa de vegetação para continuidade dos experimentos.

Aos técnicos do laboratório, em especial a Mityo, pela disponibilidade e pelo cuidado com o laboratório.

Aos pós-doutorandos Valcineide Tanobe e Oranys Gonzalez pela ajuda com as análises e com os experimentos.

Às minhas orientadas de iniciação científica, Natascha Amalio e Gabrielle Sextos, cuja ajuda foi essencial no desenvolvimento desta tese.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pelo financiamento do projeto e da bolsa de pesquisa.

À todos que de alguma forma contribuíram para a o desenvolvimento deste trabalho, meus sinceros agradecimentos!!!

“Diz-se que, mesmo antes de um rio cair no oceano ele treme de medo. Olha para trás, para toda sua jornada (...) e vê à sua frente um oceano tão vasto que entrar nele nada mais é do que desaparecer para sempre. Mas não há outra maneira. O rio não pode voltar (...). O rio precisa se arriscar e entrar no oceano. E somente quando ele entra no oceano é que o medo desaparece. Porque apenas então o rio saberá que não se trata de desaparecer no oceano, mas tornar-se oceano. Por um lado é desaparecimento e por outro lado é renascimento. Assim somos nós. Só podemos ir em frente e arriscar.”

Osho

RESUMO

O ácido giberélico (GA_3) é um hormônio presente em plantas, o qual apresenta grande interesse agrícola devido as propriedades relacionadas ao desenvolvimento vegetal. O presente estudo avaliou a produção do GA_3 via fermentação no estado semissólido, o desenvolvimento de formulações estáveis e os efeitos da sua aplicação. Foram utilizadas polpa cítrica (PC) e casca de soja (CS) como meio de inóculo e produção do GA_3 através da cepa *Fusarium fujikuroi* LPB06. Estudos para definição de formulações líquida e em pó foram realizados usando o extrato fermentado contendo GA_3 adicionado de aditivos selecionados. Os efeitos dos produtos obtidos foram avaliados em soja. Uma produtividade de 3.75 mg $L^{-1}h^{-1}$ de GA_3 foi obtida em frascos Erlenmeyer, usando uma mistura de PC/CS (70%/30%). Nas mesmas condições, uma produtividade de 1.66 mg $L^{-1}h^{-1}$ foi alcançada em Bioreator de Coluna de Bolhas e 2.13 mg $L^{-1}h^{-1}$ em Bioreator de Tanque Agitado. Nos estudos de formulação líquida, o preservante B (PB) e o surfactante A (SA) foram adicionados ao extrato purificado de GA_3, e mantiveram 40% e 24%, respectivamente, da atividade do GA_3 após 14 dias a 54°C. O diluente X (DX) e o diluente Y (DY) mostraram melhores efeitos quanto à estabilidade do GA_3, mantendo 62% e 76% da atividade, respectivamente (54 °C por 14 dias). A formulação líquida final do extrato de GA_3 (Gibbtec LS), ficou definida com a adição de DX (50%) e PB (2.5%), conservando 66% da estabilidade da molécula após 14 dias a 54 °C e 99% após 6 meses em condições ambiente de armazenagem. A formulação em pó do extrato contendo GA_3 (Gibbtec SP) foi composta de 10% do carreador A (CA), seco a 180 °C em spray dryer ou liofilizador, mantendo a estabilidade do pó em torno de 90% por 8 e 4 meses, respectivamente. Os estudos de germinação de sementes de soja foi conduzido por meio dos tratamentos T1- sem imersão; T2- água; T3- Progibb® 400; T4- Extrato GA_3; T5- Gibbtec LS e T6- Gibbtec SP. T6 apresentou o menor tempo médio de germinação (MGT) (3.08 dias), seguido pelos tratamentos T3 e T4 (3.41 e 3.45 dias). T6 atingiu a maior porcentagem de germinação (96%), 15% mais que o T3. Quanto ao comprimento de raiz e formação de raiz lateral, T4 e T6 promoveram os melhores resultados. O pré-tratamento das sementes com GA_3 (T3, T4 e T6) induziram o alongamento caulinar. Os efeitos da aplicação foliar dos tratamentos T1- água; T2- Progibb® 400; T3- Extrato GA_3; T4- Gibbtec LS; e T5- Gibbtec SP no desenvolvimento das plantas mostrou que os tratamentos contendo GA_3 aumentaram significativamente o tamanho do caule das plantas quando comparado ao T1. T2 apresentou o maior alongamento caulinar e matéria seca do caule (143.8 cm e 12.42 g), seguido pelo T3 (113.33 cm e 11.14 g) and T5 (105.03 cm e 10.23 g). O T1 apresentou altura de 61.60 cm e matéria seca do caule de 9.86 g. Contudo, os produtos desenvolvidos apresentam potencial para serem utilizados na agricultura para estimular o desenvolvimento das culturas.

Palavras-chave: GA_3. *Fusarium fujikuroi*. Polpa cítrica. Casca de soja. Hormônio de crescimento de plantas. Formulação do GA_3. *Glycine max*.

ABSTRACT

Gibberellic acid (GA_3) is a natural plant hormone, which has great agricultural interest due to its properties related to plant development. The present study evaluated GA_3 production using a low cost complex medium via semi-solid state fermentation (SSSF), the development of stable formulations and the effects of their application. Citrus pulp (CP) and soybean hulls (SH) were used in fermentation medium for GA_3 production through *Fusarium fujikuroi* LPB 06. Liquid and powder formulation studies were performed using the produced extract of GA_3. The effects of different formulations were tested on soybean (*Glycine max* Pampeana 20RR). GA_3 production of 451 mg L^{-1} was obtained after 120 hours (3.75 mg $L^{-1}h^{-1}$) using CP/SH mixture (70%/30%) in Erlenmeyer flasks. A productivity of 1.66 mg $L^{-1}h^{-1}$ was achieved in Bubble Column Reactor (BCR), and 2.13 mg $L^{-1}h^{-1}$ in Stirred Tank Reactor (STR). Liquid formulation studies were carried out with the addition of PB and SA to the purified GA_3 extract, maintaining 40% and 24% of GA_3's activity, respectively, after 14 days at 54 °C. DX (50% and 90%) and DY (90%) showed better effects on GA_3 stability, maintaining 62%, 63% and 76%, respectively, of its activity under accelerated conditions (54 °C during 14 days). The final liquid formulation of the GA_3 extract, named Gibbtec LS, was defined with the addition of DX (50%) and SA (2.5%), which promoted a conservation of 66% of the molecule's stability after 14 days at 54 °C and 99% after 6 months under ambient storage conditions. The powder formulation of GA_3 extract (Gibbtec SP) was composed of 10% CA, dried at 180 °C in a spray dryer, with a GA_3 stability around 90% after 8 months. In an alternative drying process using lyophilizer, the GA_3 extract with addition of 10% CA remained stable for 4 months. Soybean germination studies were conducted using the treatments: T1- without immersion; T2- water; T3- Progibb® 400; T4- GA_3 Extract; T5- Gibbtec LS and T6- Gibbtec SP. T6 presented the lowest mean germination time (MGT) (3.08 days), followed by treatments T3 and T4 (3.41 and 3.45 days). Treatments T1, T2 and T5 showed the highest MGT, 4.25, 4.32 and 4.79 days, respectively. T6 reached the highest germination percentage (96%), 15 % more than T3. Regarding root length and lateral root formation, T4 and T6 promoted the best results. Pre-treatment of seeds with GA_3 (T3, T4 and T6) induced caulinar elongation. The effects of foliar applications of T1- water; T2- Progibb® 400; T3-GA_3 extract; T4- Gibbtec LS; and T5- Gibbtec SP in plant development showed that treatments containing GA_3 significantly increased plant stem size when compared to control (T1). T2 presented the highest stem length and shoot dry matter (143.8 cm and 12.42 g), followed by T3 (113.33 cm and 11.14 g) and T5 (105.03 cm and 10.23 g). T1 presented 61.60 cm of plant height and shoot dry matter of 9.86 g. However, the products developed have the potential to be used in agriculture for crop improvement.

Keywords: GA_3. *Fusarium fujikuroi*. Citrus pulp. Soybean hulls. Plant growth hormone. GA_3 formulation. *Glycine max*.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATION AND ACRONYMS

BCR	Bubble collumn reactor
CP	Citrus pulp
CP/SHAE	Aqueous extract of citrus pulp and soybean hulls
CPS	*ent*-copalyl diphophate synthase
EE	Encapsulation efficiency
ELMs	Emulsion liquid membranes
ES	Fermented extract solid contente
GA	Gibberellins
GA_3	Gibberellic acid
GGPP	Geranylgeranyl diphophate
Gibbtec LS	GA_3 extract liquid solution formulation
Gibbtec SP	GA_3 extract soluble powder formulation
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
HPLC-ESIMS/MS	Liquid chromatography electrospray tandem mass spectrometry
HPLC-FD	High performance liquid chromatography coupled with fluorescent detection
HPLC-MS	High performance liquid chromatography coupled with mass spectrometry
IBGE	Brazilian Institute of Geography and Statistics
KAO	*ent*-kaurenoic acid oxidase
KO	*ent*-kaurene oxidase
KS	*ent*-kaurene synthase
MD	Maltodextrin
MEKC	Micellar electrokinetic chromatography
MF	Microfiltration
MGT	Mean germination time
n	Number of germinated seeds
PDA	Potato dextose agar
PEG	Polyethylene glycol
PGR	Plant growth regulator

R1	Soybean reprodutive stage 1
R4	Soybean reprodutive stage 4
SDS	Sodium dodecyl sulfate
SH	Soybean husk
SmF	Submerged fermentation
SSF	Solid-state fermentation
SSSF	Semi-solid state fermentation
STR	Stirred tank reactor
t	Time of germination test
TDA	Trydodecylamine
TS	Total solids
UF	Ultrafiltration
UPLC	Ultra performance liquid chromatography
V3	Soybean vegetative stage 3
Vol	Volume of fermented extract
Wt	Powder weight
$W_f(GA_3)$	GA_3 final weight
$W_{t0}(GA_3)$	GA_3 weight before drying process
$[GA_3]_f$	GA_3 concentration after drying

SUMMARY

CHAPTER 1- GENERAL ASPECTS OF GIBBERELLIC ACID

1.1 INTRODUCTION

Gibberellic acid (GA_3) is an important plant growth homone discovered in the 1930's by Japanese scientists. This hormone belongs to the gibberellins family that comprises around 140 molecules, in which GA_3, GA_4 and GA_7 show important biological properties and are commercially available. Their importance for agriculture is linked to their effect on seeds and plants development and crop quality improvement.

GA_3 is obtained by fermentation, since the chemical synthesis is economically unviable (RADEMACHER, 2015). The commonly used strain is *Gibberella fujikuroi* (renamed *Fusarium fujikuroi*), which produces GA_3 through its secondary metabolism. Different fermentation techniques have been studied, such as submerged (SmF), semi-solid (SSSF) and solid state (SSF) fermentation. The production of GA_3 by these techniques ranges from 25 mg L^{-1} ~ 3.9 g L^{-1} by SmF, 250 mg kg^{-1} ~ 6.8 g kg^{-1} by SSF, and Oliveira et al. (2017) achieved a GA_3 production of 4.8 g kg^{-1} by SSSF.The advantage of using SSSF and SSF techniques is the possibility of using agro-industrial residues or subproducts as culture medium. Different residues have been studied for this purpose, for example, citrus pulp (OLIVEIRA et al., 2017), cassava (TOMASINI; FAJARDO, 1997), coffee husk (MACHADO et al., 2002), and vegetal oil (RIOS-IRIBE; HERNÁNDEZ-CALDERÓN; ESCAMILLA-SILVA, 2016). Moreover, it is reported that the use of plant extracts in the culture medium could stimulate GA_3 production (RADEMACHER, 1994; RIOS-IRIBE et al., 2011).

GA_3's downstream processes are still little studied and reported. The most employed recovery process are adsorption and liquid-liquid extraction. In drying process, spray dryer is the oldest and well-established method to dry huge quantities of material as well as it is a low cost technology and easiness of industrialization (ANANDHARAMAKRISHNAN; RIELLY; STAPLEY, 2008; KARTHIK; ANANDHARAMAKRISHNAN, 2013). However, no reports were found about GA_3 drying in spray dryers, but Devisetty et al. (2007) studied GA_3 formulation and drying by extrusion.

China and United States are the biggest suppliers of GA_3. Its production is around 100 ton/year, in which approximately 75% of this is used for plant development,

and the rest for malting (RADEMACHER, 2015). Its large application is still limited due to its high production cost. Therefore, the development of a competitive product must take into account an economic and sustainable production with a strategy to increase its market availability and turn it accessible for a big variety of crop cultures.

1.2 OBJECTIVES

1.2.1 General objectives

The objective of this work was to produce stable liquid and powder products containing gibberellic acid (GA_3), obtained by semi-solid state fermentation using low cost fermentation medium and evaluated the effects of their application in plant development.

1.2.2 Specific objectives

a) Production of GA_3 through semi-solid state fermentation using the alternative susbtrates in Erlenmeyer flasks, and in bubble column and STR bioreactors using synthetic and alternative mediums.
b) To screen diluents and additives to compose GA_3's liquid formulation and to evaluate its stability;
c) To study the best drying conditions to obtain a powder GA_3 formulation using spray dryer and to evaluate its stability;
d) To apply GA_3's liquid and powder formulations in soybean seeds and to evaluate their effect on germination;
e) To evaluate the effect of GA_3's products application on soybean seedlings development.

CHAPTER 2 - CURRENT ADVANCES IN GIBBERELLIC ACID (GA_3) PRODUCTION, PATENTED TECHNOLOGIES AND POTENTIAL APPLICATIONS

Marcela C Camara[1]; Luciana P S Vandenberghe[1*]; Cristine Rodrigues[1]; Juliana de Oliveira[1]; Craig Faulds[2]; Emmanuel Bertrand[2]; Carlos R Soccol[1]

[1]Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, Brazil, Centro Politécnico, CP 19011, Curitiba-PR, 81531-908, Phone number: 005541 33613697

[2]Aix-Marseille Université, POLYTECH Marseille, UMR 1163 Biotechnologie des Champignons Filamenteux, 163 Avenue de Luminy, 13288 Marseille Cedex 09, France

*Corresponding author, e-mail: lvandenberghe@ufpr.br, 005541 33613697

ABSTRACT

Gibberellins consist of a large family of plant growth hormones discovered in the 1930s, which are synthesized via the terpenes route from the geranylgeranyl diphosphate and feature a basic structure formed by an *ent*-gibberellane tetracyclic skeleton. Among them, only four have biological activity, including gibberellic acid (GA_3), which acts as a natural plant growth regulator, especially for stem elongation, seed germination, and increased fruit size. It can be obtained from plants, fungi, and bacteria. There are also some reports about microalgae GA_3 producers. Fungi, especially *Fusarium fujikuroi,* are preferred for GA_3 production via submerged fermentation (SmF) or solid-state fermentation (SSF). Many factors may affect its production, some of which are related to the control and scale-up of fermentation parameters. Different GA_3 products are available on the market. They can be found in liquid or solid formulations containing only GA_3 or a mixture of other biological active gibberellins, which can be applied on a wide variety of cultivars, including crops and fruits. However, the product's cost still limits its large and continuous application. New low-cost and efficient GA_3 production alternatives are surely welcome. This review deals with the latest scientific and technological advances on production, recovery, formulation, and applications of this important plant growth hormone.

Keywords: Plant growth regulators. *Fusarium fujikuroi.* Submerged fermentation. Downstream. Formulation

2.1 INTRODUCTION

Gibberellic acid (GA_3) is a diterpenoid carboxylic acid that belongs to the gibberellins family and acts as a natural plant growth hormone. Plants and some microorganisms, such as fungi and bacteria, produce it. GA_3 has promising applications in the agro-industrial sector due to its properties related to plant development. Since it was discovered, studies have been focused on enhancing its process yield, boosting its productivity, and reducing its cost, which sometimes restricts the use of this important growth hormone.

GA_3 is applied to crops, orchards, and ornamental plants, where it plays a role in seed germination (FINCH-SAVAGE; LEUBNER-METZGER 2006; CHEN; KUO; CHIEN, 2008; URBANOVA; LEUBNER-METZGER 2016), response to abiotic stress (COLEBROOK et al., 2014), fruit growth enhancement (LI et al., 2011), stem elongation (DAYAN et al., 2012; WANG et al. 2017), flowering (SHARMA; SINGH, 2009; MUÑOZ-FAMBUENA et al., 2012), the malting of barley (BRIGGS, 1963), and other physiological effects that occur in its interaction with other phytohormones (STEFFENS; WANG; SAUTER, 2006; HEDDEN; SPONSEL 2015). This high-value molecule is mainly produced via submerged fermentation (SmF) through the secondary metabolism of the fungus *Fusarium fujikuroi*. However, its industrial recovery and formulation are still little reported.

This review focuses on general aspects of GA_3 production, the discussion about the main recovery alternatives and formulation processes, its market availability, and the biological effects in plants.

2.2 GENERAL ASPECTS

Gibberellins (GAs) were discovered in Japan in the 1930s when a group of scientists and farmers began to study a disease affecting rice fields. This disease was characterized by excessive stem growth, yellowing of the affected parties, and a lack of seed production (TAIZ; ZEIGER, 2009). GAs can possess a skeleton with 20 carbon atoms (C_{20}-GAs) or 19 carbon atoms (C_{19}-GAs), in which all biologically active GAs possess 19 carbon atoms (TARKOWSKÁ et al. 2014). Currently, around 140 known different GA molecules have been isolated from plants and microorganisms, but only

a part of them are biologically active, whereas most of them are precursors of minor importance (MACMILLAN, 2001; KAWAIDE, 2006). Those with the highest biological properties and that are commercially available are GA_3, GA_4, and GA_7.

GA_3 ($C_{19}H_{22}O_6$, CAS 77-06-5, MM: 346.37) is chemically characterized as a tetracyclic dihydroxy-γ-lactone acid containing C1-C2 double bond, C10 γ-lactone ring and a OH group in C13 (Figure 2.1a). It is a white crystalline powder with a melting point of around 233-235 °C that is soluble in alcohol, acetone, ethyl acetate, and butyl acetate, and it is sparingly soluble in petroleum ether, benzene, and chloroform. GA_3's ability to dissolve in water is low, reaching only 5 g L^{-1}. It is stable in dry conditions and readily decomposes at high temperatures, at alkaline pH values, and in aqueous solutions. Its half-life in aqueous solutions is approximately 14 days at 20 °C and two days at 50°C (BRUCKNER et al., 1991; RODRIGUES et al., 2012a). Therefore, the lack of stability can be associated with a C1-C2 double bond in its chemical structure, making the molecule more reactive. According to Albermann et al. (2013), the same occurs with GA_7 (Figure 2.1c), whereas GA_4 (Figure 2.1b) is the most stable of the three GAs. It has also been suggested that the loss of the γ-lactone ring can lead to GA_3 biological inactivation (PÉREZ et al., 1996), and therefore, the presence of γ-lactone ring and C1-C2 double bond in GA_3 structure are crucial for maintaining its biological activity.

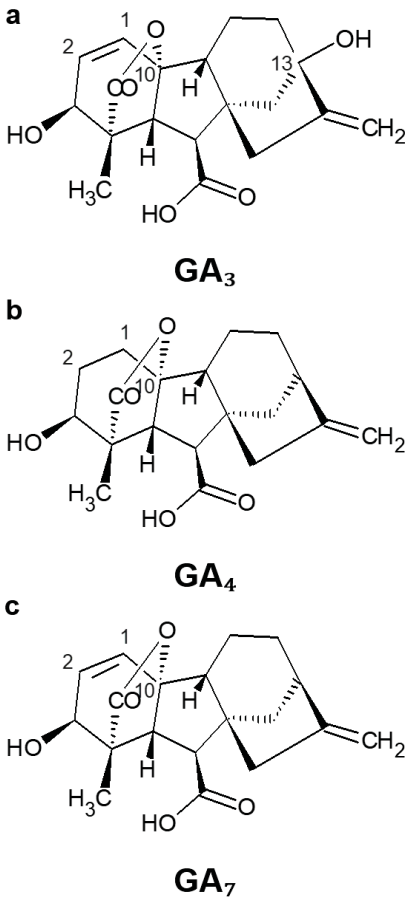


Figure 2.1 - Chemical structures of the bioactive gibberellins GA_3 (gibberellic acid) (a), GA_4 (b) and GA_7 (c).
Source: author

In aqueous solutions, GA_3 hydrolysis to gibberellenic acid may occur in a strictly chemical process; however, in the presence of microorganisms, GA_3 can be metabolized during C-limitation (HOLLMANN et al., 1995a). Depending on the temperature, the time of the reaction, and the pH of the solution, different GA_3 decomposition products can be obtained, and its “gibberellin-like” biological activity can be reduced or completely lost (PÉREZ et al., 1996). These compounds are shown in Figure 2.2.

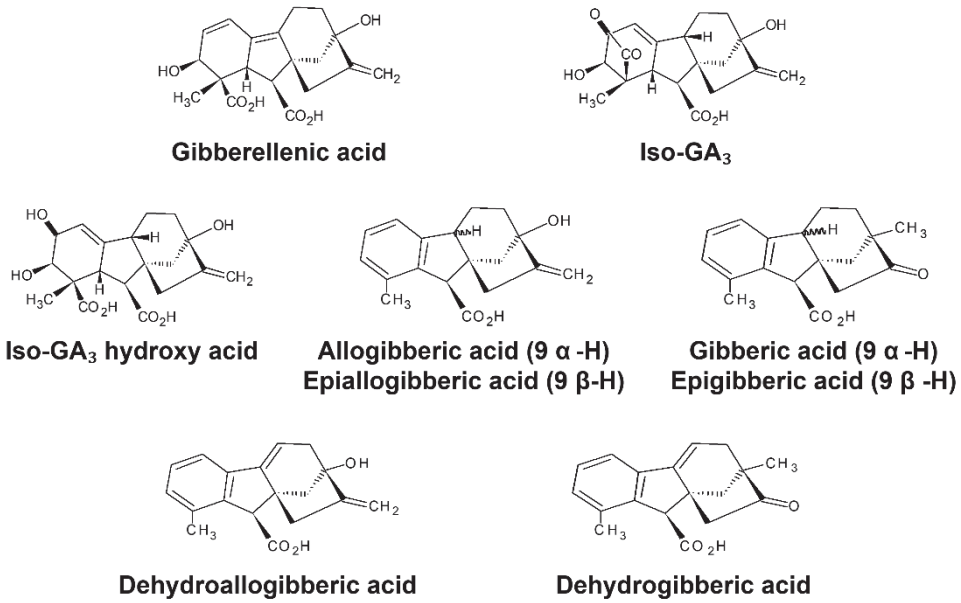


Figure 2.2 - Compounds formed from GA_3 decomposition in aqueous solution under different conditions of pH, temperature, or reaction time.
Source: author

2.3 BIOSYNTHESIS

GA synthesis occurs via the terpenes route from geranylgeranyl diphosphate by plants and by fungi. Different researchers have extensively discussed this route. For this reason, GA synthesis will be shortly discussed in this review. More information can be seen in the work of Hedden and Phillips (2000); Tudzynski (2005); Yamaguchi (2008); Bömke and Tudzynski (2009); Hedden and Sponsel (2015). The stages of GA synthesis are described below.

Stage 1. Geranylgeranyl diphosphate conversion to *ent*-kaurene

Four isoprenoid molecules are linked together to form a linear molecule of 20 carbon atoms, known as geranylgeranyl diphosphate (GGPP). This molecule is transformed into *ent*-copalyl-diphosphate through the action of an *ent*-copalyl diphosphate synthase (CPS), which in turn is converted to a tetracyclic compound known as *ent*-kaurene through the action of an *ent*-kaurene synthase (KS).

Stage 2. Ent-kaurene conversion to GA_{12}

The *ent*-kaurene oxidase (KO) in plants and the P450-4 in fungi catalyze the sequential oxidation in C-19 of *ent*-Kaurene to produce *ent*-kaurenoic acid, which is

subsequently converted to GA_{12}-aldehyde through the action of an *ent*-kaurenoic acid oxidase (KAO) in plants, and P450-1 in fungi.

Stage 3. GA_{12} conversion to other GAs

In plants, GA_{12}-aldehyde is initially converted to GA_{12} and then, through the action of GA_{20}-oxidase, which is responsible for the production of C_{19}-GAs, it is converted to GA_9. In a parallel pathway, GA_{12} is also 13-hydroxylated for the production of GA_{53}, which is converted to GA_{20} through the action of C_{20}-oxidase. Then, GA_3-oxidase converts GA_{20} and GA_9, by the addition of a 3β-hydroxyl group, into GA_1 and GA_4, respectively. GA_3 is synthesized through the conversion of GA_{20} into GA_5, by GA_3-oxidase. This stage differs between species and depends on environmental conditions.

In fungi, GA_{12}-aldehyde is 3β-hydroxylated to GA_{14}-aldehyde, which is oxidized to form GA_{14}. This last one, in turn, is converted to GA_4 through the oxidation of C20. The GA_4 is the first bioactive molecule that is formed and is desaturated to form GA_7, which is then converted to GA_3, by 13-hydroxylation. GA_1 is formed by the 13-hydroxylation of GA_4. The GA_3 biosynthesis route is described in Figure 2.3.

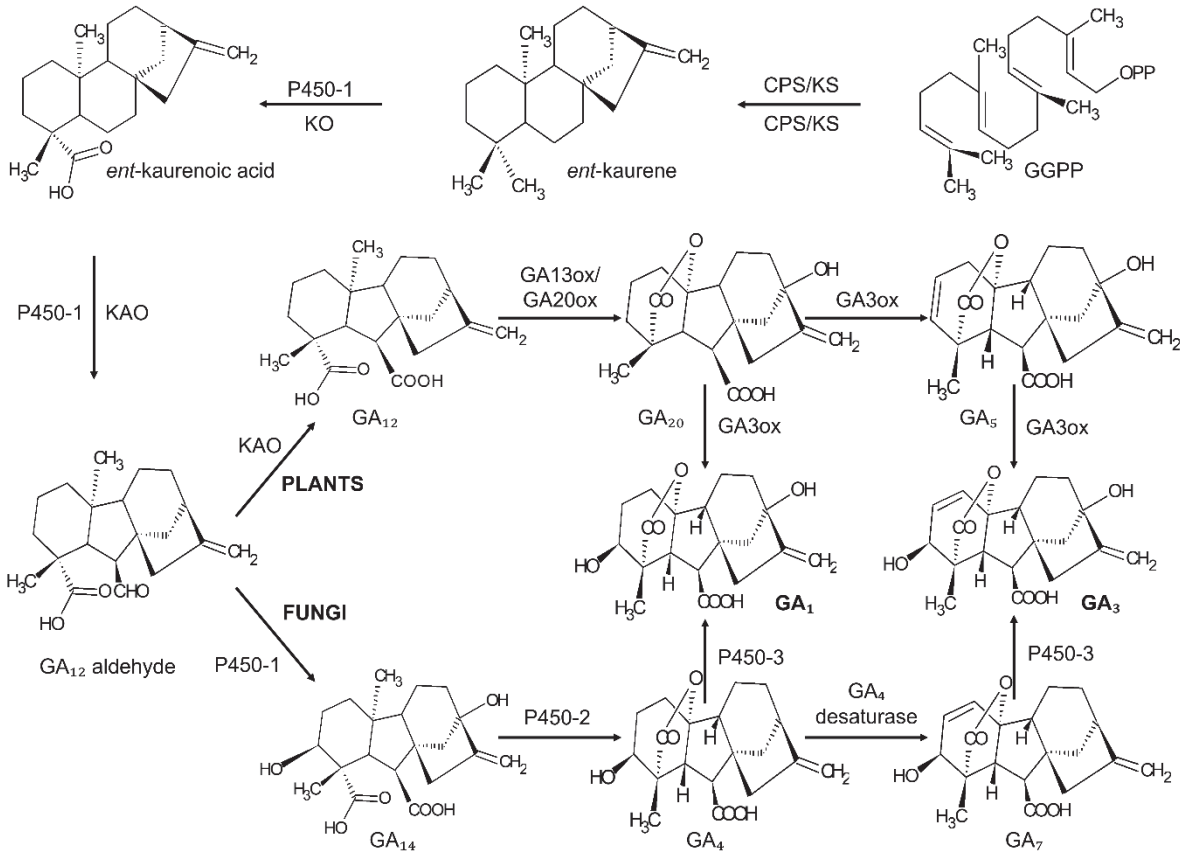


Figure 2.3 - Gibberellins (GA) biosynthetic pathway in fungi and plants. The biosynthesis starts from GGDP (geranylgeranyl diphosphate) in both, fungi and plants. The route differs from GA_{12} aldehyde, where the first GA formed is GA_{14} and GA_{12} in fungi and plants, respectively. The enzymes involved are CPS (ent-copalyl diphosphate synthase), KS (ent-kaurene synthase) and P450 (cytochrome P450 oxidoreductase) in fungi, and CPS, KS, KO (ent-kaurene oxidase), KAO (ent-kaurenoic acid oxidase) and GA oxidase (GA13ox, GA20ox and GA3ox) in plants.
Source: author

The biosynthesis pathways in plants and fungi during conversion of geranylgeranyl diphosphate to *ent*-kaurene, and subsequent conversion to GA_{12}-aldehyde, are similar. The pathways differ from the stage in which GA_{12}-aldehyde is converted to other GAs due to the order in which the steps of 3β-hydroxylation and 13-hydroxylation occur in plants and fungi.

2.4 GA_3 PRODUCTION

Since 1935, the low amounts of GA_3 obtained from plants stimulated the production of this hormone through fermentation. Since then, efforts have been made to optimize its production, reduce costs, increase productivity, and discover new ways of production. In this way, solid-state fermentation (SSF) and SmF usually stands out in GA_3 production. SSF is a process in which the microorganism grows on a solid matrix

in the absence of free water that serves as a support/substrate for microorganism development. Water is present only in a small amount that is sufficient for microorganism growth (SINGHANIA et al., 2009).

SSF shows as advantages high product yields, low bacterial contamination, less wastewater generation, easier product recovery, and low-cost agro-industrial sub-products that can be used as substrates (PANDEY; SOCCOL; MITCHELL, 2000; SELVARAJ; MURTY, 2017). However, SSF scale-up is difficult due to problems with the control of fermentation parameters. Some alternative substrates were studied for GA_3 production through SSF, such as coffee husks (MACHADO et al., 2002), citrus pulp (RODRIGUES et al., 2016; OLIVEIRA et al., 2017), and others (SATPUTE; SHARMA; MURARKAR, 2010). They are presented in Table 2.1.

SmF is the conventional and preferred technique, though it requires higher amounts of water, energy, and space, as well as higher costs of product recovery. The microorganism is cultivated in a liquid medium, usually a synthetic medium. Alternative carbon sources such as rice flour (ESCAMILLA et al., 2000; UTHANDI; KARTHIKEYAN; SABARINATHAN, 2010), corn oil (RIOS-IRIBE; HERNÁNDEZ-CALDERÓN; ESCAMILLA-SILVA, 2016), and citrus pulp (OLIVEIRA et al., 2017) have been used to compose the SmF medium for GA_3 production (Table 2.1).

Semi-solid state fermentation (SSSF) is an unusual technique and little studied for GA_3 production. This technique overcomes some drawbacks presented by SSF and SmF, especially related to heat and mass transfer, nutrient availability, and production cost. In this case, the microorganism is cultivated in a liquid medium with solids in suspension (SELVARAJ; MURTY, 2017). Agro-industrial sub-products can be used as substrate, promoting a support for fungi development and cost reduction. SSSF has been studied in the production of organic acids (MAI; LEE; CHOI, 2016), enzymes (GONZALEZ et al., 2013; SELVARAJ; MURTY, 2017), and single cell oil (ECONOMOU et al., 2010).

Recently, Oliveira et al. (2017) studied the effect of different fermentation systems for GA_3 production using low agro-industrial sub-products as substrates. The authors achieved higher production using SSF (946 mg L^{-1}; 7.60 g kg^{-1} dry citrus pulp) and SSSF (331 mg L^{-1}; 7.69 g kg^{-1} dry citrus pulp) than using an aqueous extract of citrus pulp via SmF (236 mg L^{-1}; 2.74 g kg^{-1} dry citrus pulp) in Erlenmeyer flasks. SSSF

showed positive results regarding GA_3 production, and therefore, new studies with this technique may lead to its higher production with lower costs.

Table 2.1 - Production of GA_3 using different culture medium and fermentation strategies.

Microorganism	Fermentation	Substrate	Production	Time	References
Fusarium moniliforme	SmF with immobilized cells	Glucose	680 mg L^{-1}	12 days	KAHLON AND MALHOTRA (1986)
Fusarium fujikuroi	SSF	Wheat bran	1.2 g kg^{-1}	7 days	KUMAR AND LONSANE (1990)
Fusarium fujikuroi	SSF	Wheat bran	6.8 g kg^{-1}	8 days	AGOSIN et al. (1997)
Fusarium fujikuroi	SSF- Fed-batch operation	Wheat bran	3 g kg^{-1}	10 days	BANDELIER et al. (1997)
Fusarium fujikuroi	SmF	Glucose and lactose	25 mg L^{-1}	5 days	TOMASINI AND FAJARDO (1997)
	SSF	Cassava	250 mg kg^{-1}	1.5 days	
Fusarium fujikuroi	SmF	Glucose and rice flour	1.100 mg L^{-1}	-	ESCAMILLA SILVA et al. (1999)
Fusarium fujikuroi	SmF with immobilized cells	Glucose and rice flour	3.900 mg L^{-1}	8 days	ESCAMILLA et al. (2000)
Fusarium fujikuroi	SSF	Coffee husk	492 mg kg^{-1}	7 days	MACHADO et al. (2002)
Fusarium fujikuroi	SmF- Fed-batch operation	Glucose	1.680 mg L^{-1}	4 days	SHUKLA et al. (2005)
Fusarium moniliforme	SSF	Citrus pulp	5.9 g kg^{-1}	3 days	RODRIGUES et al. (2009)
Fusarium fujikuroi	SmF	Glucose and rice flour	1.175 mg L^{-1}	7 days	UTHANDI et al. (2010)
Mutant strains of *Fusarium fujikuroi*	SmF- Fed-batch operation	Glucose and wheat gluten	600 mg L^{-1} of GA_4	7 days	LALE AND GADRE (2010)
Fusarium proliferatum	SSF	Pigeon pea pods	7.8 mg g^{-1}	8-10 days	SATPUTE et al. (2010)
		Sorgum straw	5.5 mg g^{-1}		
		Corncobs	6.1 mg g^{-1}		
Fusarium fujikuroi	SmF	Glucose and corn oil	380 mg L^{-1}	12 days	RIOS-IRIBE et al. (2011)
Fusarium moniliforme	SmF	Glucose	15 g L^{-1}	10 days	RANGASWAMY (2012)
	SSF	Jatropha seed cake	105 mg g^{-1}	4 days	
Mutant strain of Fusarium fujikuroi	SmF	Glucose	216 mg L^{-1}	10 days	ALBERMANN et al. (2013)
Fusarium fujikuroi	SmF	Corn oil	360 mg L^{-1}	12 days	RIOS-IRIBE et al. (2016)
Fusarium fujikuroi	SSSF	Citrus pulp	4.8 g kg^{-1}	9 days	OLIVEIRA et al. (2017)

Source: author

2.5 FACTORS AFFECTING GA_3 PRODUCTION

2.5.1 Medium composition and operation systems

Whichever fermentation method is used, the chemical and physical conditions are crucial factors for the development of the microorganism and, consequently, the production of its metabolites. Among the nutrients that most affect GA_3 production, carbon and nitrogen sources are the most important. Glucose and sucrose are commonly used as carbon sources for GA_3 production, though mannitol, maltose, starch, and glycerol are also reported (KAHLON; MALHOTRA, 1986; KUMAR; LONSANE, 1990; PASTRANA et al., 1995; TOMASINI; FAJARDO, 1997; MACHADO et al., 2001, 2002; RODRIGUES et al., 2012a).

The presence of nitrogen in the medium is important for GA production due to regulatory processes involving ammonium. However, GA_3 synthesis starts only when the exhaustion of nitrogen occurs in the medium (BRUCKNER et al., 1991; ESCAMILLA SILVA et al., 1999; TUDZYNSKI, 2005). Nitrogen sources such as ammonium sulfate, ammonium chloride, glycine, ammonium tartrate, corn steep liquor, and plant oil are preferred. Thus, the C:N ratio normally used to get a better yield of GA_3 is between 6:1 and 45:1, but C:N can be different, from 10:1 to 25:1 in the early stages, and from 25:1 to 200:1 in the final stages (KUMAR; LONSANE, 1990; BRUCKNER et al., 1991; MACHADO et al., 2001; RODRIGUES et al., 2012a). This fact reveals the viability of fed-batch operations, in which it is possible to feed the medium with carbon sources during the GA_3 production process.

There are some reports about fed-batch operations for GA_3 production in the literature. Pastrana et al. (1995) studied fed-batch operation using mussel processing waste as starting and feed mediums. With this operation system, these authors obtained an increase in biomass content as well as in the production of GA_3. Bandelier et al. (1997) successfully studied an SSF fed-batch system using wheat bran as the solid medium and cornstarch as feed solution. Shukla et al. (2007) produced GA_3 trough SmF and used glucose to feed the medium. They reached 3.24 g of GA_3 total mass in fed-batch operation, almost two times greater than in the batch process (1.8 g

of GA_3 total mass). Lale and Gadre (2010) reported a GA_3 production of 113 mg L^{-1} in fed-batch fermentation after feeding the medium with glucose.

The use of agro-industrial residues as carbon or nitrogen sources was extensively reported for GA_3 production with different fermentation systems (MACHADO et al., 2002; RODRIGUES et al., 2009a; SATPUTE; SHARMA; MURARKAR, 2010; RANGASWAMY, 2012; RIOS-IRIBE; HERNÁNDEZ-CALDERÓN; ESCAMILLA-SILVA, 2016; OLIVEIRA et al., 2017). This practice surely provides environmentally friendly recycling, reducing the environmental impact, and enables a possible reduction of the final product cost. These residues generally present all sources of nutrients required for microorganism growth (PANDEY; SOCCOL; MITCHELL, 2000; SINGHANIA et al., 2009). Soybean, sugar beet, sweet potato, potato, and sorghum residues; wheat and rice straw; corn, rice, and soybean hulls; and sugarcane and cassava bagasse can be used as substrates for GA_3 production. Processing waste from the coffee industry, fruit industries, and oil mills is also employed (PANDEY et al. 2000; SOCCOL AND VANDENBERGHE 2003; SINGHANIA et al. 2009).

2.5.2 Physical factors

Physical factors affecting GA_3 production are temperature, pH, agitation, aeration, water activity, and/or humidity (SINGHANIA et al., 2009; RIOS-IRIBE et al., 2011). The optimum temperature varies from 25 °C to 32 °C depending on the used strain. The optimal pH for GA_3 production is the generally employed range of 3.5 - 5.8 (RODRIGUES et al., 2012a).

Aeration is surely necessary because the route of the biosynthesis of GAs involves a series of oxidative steps. Therefore, the microorganism's demand for oxygen may increase with the growth of mycelium. Agitation should allow efficient homogenization as well as promote the mass transfer inside the fermenter (MACHADO et al., 2001; TUDZYNSKI, 2005).

2.6 GA_3 SEPARATION METHODS

Separation and purification of biomolecules involve different steps depending on the characteristics and purity of the final commercial products. Downstream

processes generally comprise higher cost techniques. Usually, the recovery of biomolecules from SmF medium is more expensive and time-consuming than from SSF or SSSF mediums. The scheme shown in Figure 2.4 describes some possible ways to recover GA_3 from SSF (BANDELIER; RENAUD; DURAND, 1997; SATPUTE; SHARMA; MURARKAR, 2010; RODRIGUES et al., 2016; OLIVEIRA et al., 2017) and SmF (LU; XIE; KUMAKURA, 1995; ATES; OZENIR; GÖKDERE, 2006; MELEIGY; KHALAF, 2009; RIOS-IRIBE et al., 2011) fermented mediums.

Generally, GA_3 recovery from SSSF and SmF starts with filtration or centrifugation steps to remove biomass and larger particles. Then, the liquid fraction is subjected to steps of adsorption, liquid-liquid extraction, or clarification. Repeated liquid-liquid partition followed by vacuum concentration is the most employed technique. For solid samples, the main step consists of extraction with solvents, but two other techniques could be used, such as supercritical fluid extraction or multiple countercurrent leaching (KUMAR; SANKAR; LONSANE, 1991; SHUKLA; SRIVASTAVA; CHAND, 2003). Nevertheless, these techniques can make the downstream process more expensive and involve the use of toxic solvents.

SmF is the main technique employed in industrial production of GA_3 by the fungus *F. fujikuroi*. However, the GA_3 downstream industrial process is still unclear. Adsorption and liquid-liquid extraction are simple techniques that can be industrially applied to recover GA_3 from liquid extracts. The separation and concomitant purification through membranes are also applicable, but still little reported for GA_3 clarification and recovery.

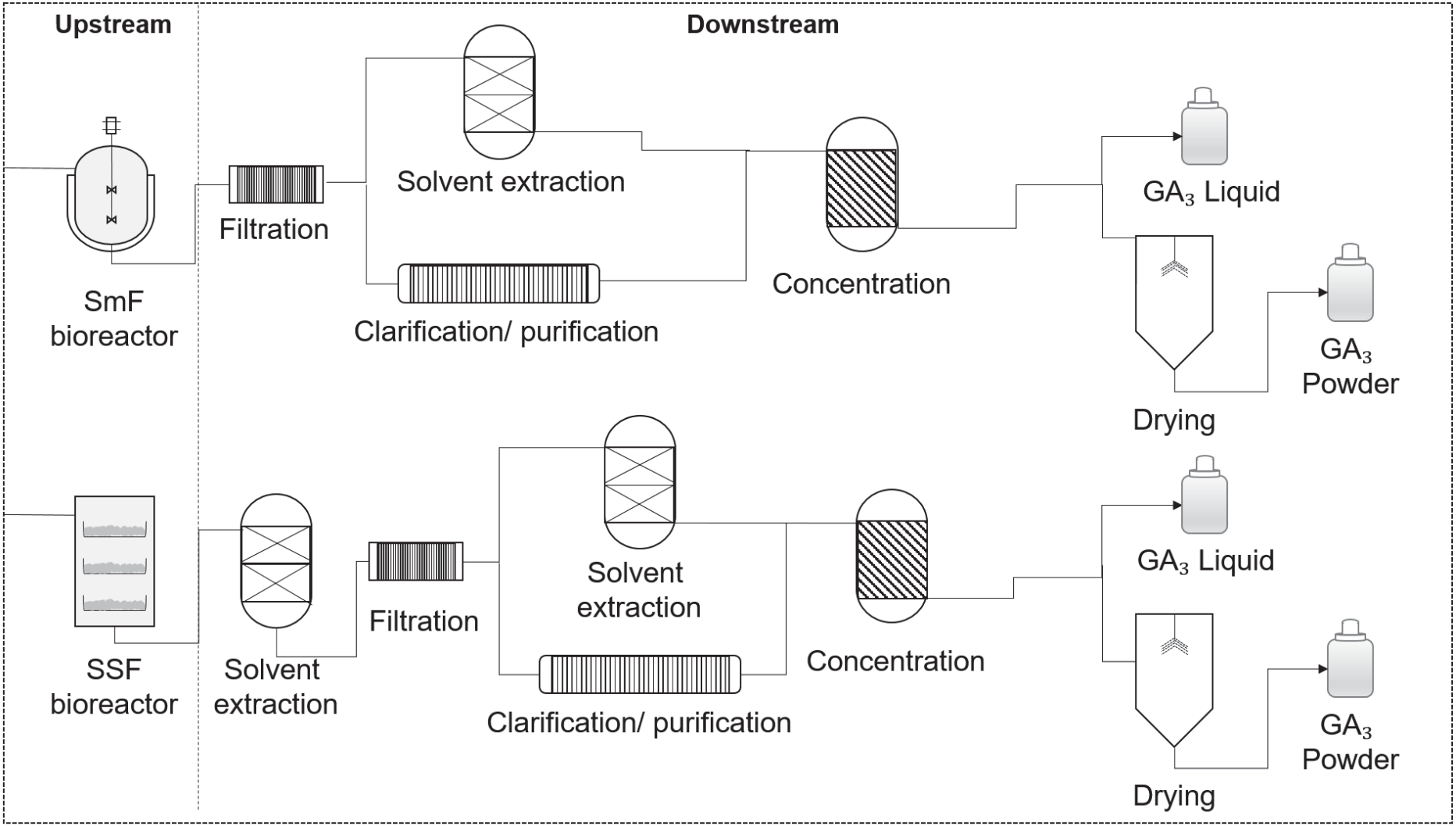


Figure 2.4 - Biotechnological process of gibberellic acid (GA_3) production via submerged fermentation (SmF) and solid state fermentation (SSF), and its separation and formulation processes to obtain the bioproduct.
Source: author

2.6.1 Adsorption

Adsorption is commonly used in industrial purification and separation of biomolecules. The process uses an adsorbent column, which is packed with a solid resin with an affinity to the solute, to separate the desired biomolecule. Then, the biomolecule can be recovered by the loaded resin, while other components of the solution flow through the system (MAGALHÃES et al., 2017). The biomolecule of interest is then recovered by elution with another solvent.

Adsorption columns have been employed in the recovery of GA_3. It is a simple, industrially applicable process, in which it is possible to reduce energy consumption and GA_3 loss. Tang et al. (2000) compared the adsorption efficiency between medium and weak polarity (X-5, S-8, and AB-5) resins with nonpolar (D3520, D4006, and D4020) and polar (NKA-9) resins. Due to the low polarity of the GA_3 molecule, the resins with moderate polarity showed larger adsorption capacities. GA_3 recovery reached 90% using S-8 resin, and the concentration was seven times greater than before the adsorption and desorption. Another advantage is that the resin can be reused for other cycles.

Wang et al. (2008) showed similar results, in which GA_3 adsorption capacity in medium polar resins was relatively higher than in nonpolar resins and lowest in the strong polar resins. GA_3 recovery can reach 95% using methanol as eluent. Rodrigues et al. (2016) used XAD-16, C18, and active charcoal to clarify the GA_3 extract obtained after SSF. The authors reported a recovery of 35.2% using XAD-16 and 42.6% using C-18. The recovery with active charcoal reached 63% of GA_3 from the extract. Active charcoal is an absorbent material that can absorb pigments from an extract and promote its clarification (ATES; OZENIR; GÖKDERE, 2006). This technique was employed and patented by Rodrigues et al. (2012b) for GA_3 fermented medium clarification.

2.6.2 Liquid-liquid extraction

Solvent extraction consists of passing the analyte of interest from a liquid phase (usually aqueous) to another liquid phase (usually organic) in which it has more affinity (higher solubility or chemical interaction). The most used solvent for GA_3

extraction is ethyl acetate, to which GA_3 has more affinity than water (ESCAMILLA et al., 2000; BERRIOS; PYLE; AROCA, 2010). However, Uslu et al. (2014) evaluated GA_3 extraction via trydodecylamine (TDA) dissolved in three different solvents and obtained a maximum extraction of 96.37% with TDA and isoamylalcohol.

Uslu (2012) evaluated the reactive extraction for GA_3 to overcome the low extractability of most organic acids by current solvents. Reactive extraction uses an extractant to remove the acid. Long-chain aliphatic tertiary amines (anion exchange extractants) with seven to nine carbon atoms in each alkyl group are the most effective and most-used extractants for carboxylic acids. The study focused on the effects of organic diluents on the extraction of GA_3 with Aliquat 336 (A336). Diluent extractions, without A336, were less effective, and isoamylalcohol + A336 was the most effective method, with 79% of GA_3 extraction.

After extraction, a concentration step is commonly carried out to evaporate the solvent from the organic phase and recover the molecule of interest (RODRIGUES, 2010). In the case of GA_3, after the evaporation step, an amorphous powder or crystalline product is obtained (BRUCKNER et al., 1991; SHUKLA; SRIVASTAVA; CHAND, 2003).

2.6.3 Membrane separation

Separation through membranes has been used for various downstream processes. However, this technique may be relatively expensive and present some operational problems. Berrios et al. (2010) found some limitations in the GA_3 separation through emulsion liquid membranes (ELMs) related to emulsion stability and water transport. Even so, 67% of GA_3 extraction from the fermented broth was reached, appearing as an alternative to the liquid-liquid extraction process.

Diafiltration and eletrodialysis are two other membrane separation methods that can be employed for organic acids recovery (MAGALHÃES et al., 2017). However, no reports were found related to GA_3 separation through diafiltration or eletrodialysis, even with ultrafiltration techniques. The search for new industrially viable GA_3 recovery techniques is still necessary, as well as the optimization of the already used techniques, seeking the reduction of downstream costs and the reduction of GA_3 losses that usually occur.

2.7 ANALYSIS AND QUANTIFICATION OF GA_3

During fermentation, different GAs are produced concomitantly with GA_3. The quantification and/or identification of GAs that are present in the fermented medium can be conducted via different methods, such as spectrophotometric, colorimetric, and fluorometric analysis (BERRÍOS; ILLANES; AROCA, 2004).

2.7.1 Spectrophotometric method

GA_3 spectrophotometric analysis at 254 nm was described by Holbrook et al. (1961). This method is simple and quick, ideal for the measurement of higher concentrations and number of samples, but it is not specific for GA_3. In this technique, GA_3 is converted into gibberellenic acid through broth acidification via hydrogen chloride (HCl). However, this method requires high purity of broth samples to avoid interferences during the quantification, especially when undefined fermentation mediums are used.

Berríos et al. (2004) proposed a method for GA_3 quantification that avoids the pre-treatment of the broth samples and, according to the authors, the advantage of this method is the fact that the conversion of GA_3 into gibberellenic acid is lineal within 2 min of reaction when HCl is added. Their method is also based on the conversion of GA_3 to gibberellenic acid through acidification, and its accuracy is above 97% for GA_3 concentration between 0.1-1 g L^{-1}. It is simple, fast, and reliable quantification for high amounts of samples.

2.7.2 Chromatographic method

Chromatographic methods is more sensitive than spectrophotometry analysis due to enables separation and identification of the different GAs produced during fermentation. High performance liquid chromatography (HPLC) with UV detection is the most used chromatographic technique to quantify GA_3 from fermented broth (ESCAMILLA et al., 2000; SHUKLA; CHAND; SRIVASTAVA, 2007; RIOS-IRIBE; HERNÁNDEZ-CALDERÓN; ESCAMILLA-SILVA, 2016; RODRIGUES et al., 2016).

However, different detectors have been employed in GA_3 quantification by liquid chromatography, such as liquid chromatography-electrospray tandem with mass spectrometry (HPLC-ESI-MS/MS) (HAO et al., 2015), HPLC coupled with fluorescence detection (HPLC-FD) (LU et al., 2016), and ultra-performance liquid chromatography (UPLC) followed by ESI-MS/MS (URBANOVÁ et al., 2013). Sharma et al. (2004) studied HPLC coupled with mass spectrometry (HPLC-MS) for GA_3 detection in fermentation broth. According to them, HPLC-MS is less time-consuming and very sensitive and selective. Moreover, sample preparation is simple.

Micellar electrokinetic chromatography (MEKC) was study as alternative method to HPLC in GA_3 determination from fermentation broths (NHUJAK et al., 2005). The authors reported as advantages of this method, for GA_3 analysis, the high accuracy and precision, shorter analysis time and it is not necessary solvent extraction, only sample filtration.

2.7.3 Fluorescence method

Fluorometric analysis quantifies the GA_3 present in the sample through the reaction with sulfuric acid (85%), in the cold. The problem related to this technique is that there is no distinction between GA_3 and gibberellenic acid, which may lead to quantification errors. Therefore, this method requires attention during sample preparation, and total fluorogen that is measured as GA_3 must be corrected for the amount of gibberellenic acid in the sample (KAVANAGH; KUZEL, 1958).

2.8 COMMERCIAL PRODUCTS CONTAINING GA_3 AS PLANT GROWTH REGULATOR

Different types of commercial formulations that are available in the market contain GA_3, or a mixture of GA_4 and GA_7. They can be obtained in liquid, soluble powder, wettable powder, tablet or water-dispersible granular forms. Some trade names of products containing GA_3 and their manufacturers are presented in Table 2.2.

Liquid formulations are associated with the relative lack of stability of GA_3 in the presence of water, which may lead to a short shelf life. Therefore, some solvents are used to minimize GA_3 degradation, but this could result in flammable formulations

that would require great care with packaging, transportation, and storage. Due to the disadvantages of liquid formulations, solid formulations seem to be a safer strategy, but they also have some drawbacks, such as dust when pouring, transferring, or measuring; the possibility of generating residues in the tank and plants; and lumps could be formed, too (DEVISETTY et al., 2007).

Table 2.2 - Different types of GA_3 products available in the international market and their manufacturers.

Plant growth hormone	Formulation type	Trademarks	Producers
GA_3	Liquid	PastureGibb®	Orion Crop Protection
		GibGro® 4LS	NuFarm Americas
		Progibb® 4% / Rizup® 4SL / Release LC	Valent BioSciences Corporation
		GibGro®	NuFarm Americas
		Gibb-gro®	Fertco
	Soluble powder/ wettable powder	Progibb® 2x/ Release®/ Berelex® 2x	Valent BioSciences Corporation
	Tablet	Progibb® tablet/ Berelex® tablet	Valent BioSciences Corporation
	Soluble granule	Express®	Ravensdown
		Progibb® SG / Progibb® 40 SG / Activol® 40SG/ Berelex® 40SG / Ryzup® 40SG/ Ryzup® SmartGrass 40WSG	Valent BioSciences Corporation
		Gibb-star®	Sum Farm New Zealand Limited

Source: author

For most commercial products, GAs are dissolved in an alcohol solution, such as isopropanol, methanol, or ethanol, at a concentration of about 4% (w/v). Some additives and/or adjuvants are also added to increase the stability of the product, targeting a protective effect of the molecule, increasing the shelf life, or increasing nutritional factors in plants (GEARY; BEACH; HABER, 1962; DATTA; VASEK; PASIETA, 2006; DEVISETTY et al., 2006; WANG et al., 2013). Some examples of

diluents, additives, or adjuvants that can be employed in a GA_3 formulation are described in Table 2.3.

Table 2.3 - Diluents, additives, and adjuvants commonly employed for GA_3 formulations.

Class	Exemples
Diluents for liquid formulation	Polyethylene glycol, ethanol, isopropyl alcohol, methyl alcohol, glycerol, propanol, n-butanol, n-amilalcohol, acetone, methyl butyl ketone, ethyl lactate, n-butyl lactate, propylene glycol
Diluents for solid formulation	Sorbitol, mannitol, lactose, dextrose, starch, limestone, sucrose, maltose, maltodextrin, and frutose
Preservatives	Sulfur dioxide, benzoic acid, sorbic acid, propionic acid, sodium and potassium salts, nitrite and nitrate of sodium and potassium
Surfactants	Nonionic: ethoxylated esters of sorbitan, fatty acid esters, glucose and sucrose esters, ethoxylated alcohols, ethoxylated alkylphenols, ethoxylated fatty acids Anionic: phosphate esters, sulfate and sulfonated oils, sulfates and sulfonates ethoxylated alkylphenols
Antioxidants	Gallate (propyl, octyl and dodecyl), ascorbic acid, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ)
Antifoam	Silicone emulsions full of silica
Encapsulating agents	Gums (arabic, alginate and carrageenan), proteins (whey and milk proteins, gelatin), carbohydrate (maltodextrin, starch, sucrose, glucose, trehalose, pectin)
UV protectant	Benzophenone-3 and ethylhexyl methoxycinnamate.
Anti-caking	Silica, talc, and limestone
Binder	Maltodextrin, lecithin and polyvinylpyrrolidone

Source: author

There are different formulations containing GA_3, which are patented by researchers and large companies. Some of them are presented in Table 2.4.

For a bioproduct formulation, the cost that will be added to its final price should be taken into account. However, the cost of additives, which are used to ensure good stability and long shelf life of the product, and the way in which the material will be packed, transported, and stored, must be evaluated.

Liquid or solid formulations can be applied through conventional ground or aerial applications. Spray volumes are variable depending upon the orchard or crop, the growth stage, and the climatic conditions. The concentration of the plant growth regulator will vary depending upon the type of fruit to be treated, the peculiarities of the locality, and the desired result (DEVISETTY et al., 2007).

Table 2.4 – Patented formulations containing GA_3.

Formulation type	Strategy	Patent number	References
Powder	Extraction and concentration of the crude extract to obtain crystals of GA_3	US 2842051	BRIAN et al. (1958)
Powder	Mixture of GA_3 salts with surfactant and diammonium phosphate	US 3004845	LA PIERRE AND ISELIN (1961)
Granular	Extrusion of a mixture of salts of GA_3 with surfactant and diammonium phosphate		
Powder	Mixture of GA_3 powder with anhydrous salts	US 3031290	SENIOR AND PARK (1962)
Powder/ liquid/ paste/ spray	Transformation of GA_3 into ester and then is added the adjuvants for each type of formulation	US 3038794	GEARY et al. (1962)
Liquid	Mixture of GA_3 with organic solvent, triglycerides, esters of fatty acids and an additional plant growth hormone	WO/2000/002454A1	RADEMACHER et al. (1999)
Granular	Mixture of GA_3 powder with an inert carrier, a disaccharide and a surfactant	US 6984609	DEVISETTY et al. (2006)
Liquid	Mixing GA_3 powder in a blend of solvents	US 0172890	DATTA et al. (2006)
Liquid	Mixture of GA_3 powder with antioxidants, surfactants, solvent and a UV protector	US 8454982	WANG et al. (2013)
Liquid	Mixture of GA_3, GA_4 or GA_7 purified with polyethylene glycol and nonionic or anionic surfactant	US 20150173365	DEVISETTY et al. (2015)
Liquid	Mixture of powdered gibberellins with adjuvants	US 20160360748	PAWLAK (2016)

Source: author

2.9 ECONOMIC ASPECTS

The demand for food by the increasing global population has sparked research on how to improve the quantity and quality of agricultural production (LIU et al., 2013). GA_3, as a plant growth hormone, plays an important economic role due to its wide range of applications, from crops to fruits. The annual production of GA_3 is around 100 tons, with a market value of US$ 100 million, from which approximately three quarters are used for plant production and the other quarter for malting (RADEMACHER, 2000; SHUKLA; SRIVASTAVA; CHAND, 2003; RANGASWAMY, 2012). Nowadays, GA_3 price for agricultural application ranges from US$ 150/kg to US$ 500/kg (prices searched in alibaba.com, accessed in 04/2018).

Worldwide application of GA_3 occurs in viticulture to improve fruit size. However, its application is not only limited to viticulture, Valent BioSciences Corp., one of the biggest providers of GAs, has listed more than 40 different crops, such as fruits, vegetables and cereals, for which GA preparations can be applied, improving quality and value (RADEMACHER, 2015). Some advantages of GA_3 application on plant development are shown in Table 2.5.

Table 2.5 - Benefits of GA_3 applications in some cultures.

Species	Benefits	References
Malting barley (*Hordeum vulgare*)	Increase of hydrolytic enzyme activity	MACLEOD AND MILLAR (1962)
Cherries (*Prunus avium* L.)	Increase in size, weight, and firmness of the fruits	HORVITZ et al. (2002)
Black mulberry (*Morus nigra* L.)	Break of seed dormancy	KOYUNCU (2005)
Cactus (*Trichocereus terscheckii*)	Seed germination	ORTEGA-BAES AND ROJAS-ARÉCHIGA (2007)
Maize plants (*Zea mays* L.)	Overcome of salt stress	TUNA et al. (2008)
Tomato (*Solanum lycopersicum* L.)	Decrease of seed germination time and greater development in field	BALAGUERA-LOPEZ et al. (2009)
Seedless grapes (*Vitis vinifera* L.)	Increase of grape bud size	CASANOVA et al. (2009)
Soybean (*Glycine max*)	Amelioration of salt stress effects and restoration of the normal development of soybeans	HAMAYUN et al. (2010)
Sugarcane (*Saccharum officinarum* L.)	Salt tolerance	SHOMEILI et al. (2011)
Helleborus niger / Helleborus x ericsmithii	Promotion of flower bud growth, increase in the number and diameters of flowers	CHRISTIAENS et al. (2012)
Tagetes patula	Bioremediation of cadmium- and benzo[a]pyrene- contaminated soils	SUN et al. (2013)
Maize (*Zea mays* L.)	Alleviation of drought effects on maize at the vegetative phase	AKTER et al. (2014)
Sunflower	GA_3 and press mud application allowed the growth of sunflower plants in Cr-contaminated soil	SALEEM et al. (2015)

Rabbiteye blueberry (*Vaccinium ashei*)	Increase of inflorescence number, and enhancement of fruit quality and plant growth	ZANG et al. (2016)
Maize plants (*Zea mays* L.)	Application of GA_3 and silicon protects, corn from *Spodoptera frugiperda* at the adult level and improves plant development	ALVARENGA et al. (2017)

Source: author

GA_3 is one of the best-selling and most important plant growth regulator (PGR). The advantages of its worldwide use is to improve crop production, quality and value. Because of the high investments and involved production costs, only few companies are still engaged in developing PGRs, reducing its large utilization. These companies still concentrate their investments on herbicides, insecticides and fungicides (RADEMACHER, 2015). However, this scenario is expected to change with the search for a more sustainable agriculture, expanding the production and use of GA_3 products.

Brazil is the most important agriculture-based economy in the world. In the last harvest (2015/2017), the Brazilian planted area was estimated at 58.17 million hectares, and the estimated grain production was 238.7 million tons (CONAB, 2016). Moreover, Brazil is the third largest world fruit producer. In 2015, fruit production reached 41 million tons according to the Brazilian Institute of Geography and Statistics (IBGE) (CARVALHO et al., 2017). The use of GA_3 in Brazilian cultivars may contribute to increasing the agricultural productivity, as well as their quality, therefore encouraging the national production and viable application of this hormone.

2.10 FUTURE PROSPECTS

GA_3 is an important plant growth regulator from the GA family. In this review, the main topics related to GA_3 production and its commercialization and application were discussed. The main bottleneck of this plant hormone's large production is correlated with its low productivity and high costs. However, different strategies of production try to overcome these problems, such as strain improvement and alternative production strategies.

Downstream processes for GA_3 recovery are generally based on solvent extraction, adsorption and concentration methods. However, adsorption and extraction through membranes can also be applied. One of the main barriers to its application is

the definition of stable liquid or solid formulations. Different GA_3 commercial products are reported and available in the world market, enabling its application in large diversity of cultivars. The search for new and low-cost techniques for GA_3 production would certainly enable its massive applicability, which would benefit the quality and productivity of different cultivars all over the world, especially in Brazil, one of the most important agriculture-based economies.

CHAPTER 3 – NEW ALTERNATIVE FOR GIBBERELLIC ACID PRODUCTION BY *Fusarium fujikuroi* USING LOW-COST AGROINDUSTRIAL SUB-PRODUCTS

Marcela Candido Camara[1]; Luciana Porto de Souza Vandenberghe[1]; Gabrielle Camille Sextos[1]; Antonio Irineudo Magalhães Jr.[1]; Cristine Rodrigues[1] and Carlos Ricardo Soccol[1]

[1] Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, Brazil, CP 19011, Curitiba-PR, Brazil, 81531-908, Phone number: 005541 33613271

ABSTRACT

Gibberellic acid (GA_3) is a natural plant growth regulator (PGR), commonly used in agriculture and horticulture products. Low cost alternative sub-products were proposed as substrate for GA_3 production by semi-solid state fermentation (SSSF). Citrus pulp (CP) and soybean hulls (SH) were employed for both inoculum and GA3 medium production by *Fusarium fujikuroi* LPB 06. A CP/SH mixture (70%/30%) promoted the production of 451 mg L^{-1} after 120 hours (3.75 mg $L^{-1}h^{-1}$). A productivity of 1.66 mg $L^{-1}h^{-1}$ was achieved in Bubble Column Reactor (BCR), and 2.13 mg $L^{-1}h^{-1}$ in Stirred Tank Reactor (STR). The cost of GA_3 fermentation medium in different scenarios showed that the use of CP/SH (70%/30%), which are being proposed in this work, reduced in 81.6% (optimistic scenario) and in 86.3% (pessimistic scenario) the medium price comparing to the synthetic medium. Therefore, the use of CP and SH through SSSF is a promising alternative for economic plant hormone production that could amplify GA_3 large application and benefit for cultivars.

Keywords: GA_3. Semi-solid state fermentation. Soybean hulls. Citrus pulp. Bubble column reactor. Stirrer tank reactor

3.1 INTRODUCTION

Gibberellic acid (GA_3) is a plant growth hormone belonging to the gibberellins (GA) family, which is involved with the development and physiological process in plants. This hormone acts in seed germination (CHEN; KUO; CHIEN, 2008); response to abiotic stress (COLEBROOK et al., 2014); fruit growth (LI et al., 2011); stem elongation (DAYAN et al., 2012); flowering (SHARMA; SINGH, 2009; MUÑOZ-FAMBUENA et al., 2012); malting of barley (BRIGGS, 1963), and other physiological effects when they interact with other phytohormones (STEFFENS; WANG; SAUTER, 2006).

Nowadays, *Fusarium fujikuroi* has been industrially employed in GA_3 production through submerged fermentation (SmF). However, semi-solid state fermentation (SSSF) technique, which involves the use of solid substrate in suspension, may be a promising way for lowering GA_3's production costs. In this method, free water content is increased in comparison with solid-state fermentation (SSF), overcoming some drawbacks presented by this fermentation technique, such as the possibility to control some parameters. Moreover, it is possible to use agricultural wastes and/or sub-products as fermentation medium components. SSSF has been studied for laccase (GONZALEZ et al., 2013), tannase (SELVARAJ; MURTY, 2017), alpha amylase (YARAŞ; SELEN; ÖZER, 2015), and oxalic acid (MAI; LEE; CHOI, 2016) production. Recently, Oliveira et al. (2017) compared GA_3 production through SSSF and SmF reaching a production of 331 mg L^{-1} and 236 mg L^{-1}, respectively.

The use of complex culture media with plant components are reported to be better for GA_3 synthesis than synthetic media composed by glucose and defined nitrogen sources. Rademacher (1994) suggested that plant extracts might contain precursors or inductors of GA synthesis, which favors GA_3 production. Moreover, the use of wastes in fermentation medium provides their environmentally friendly recycle, reducing environmental impact, besides enabling a possible reduction of process costs.

Alternative substrates have been employed in GA_3 production, such as cassava (TOMASINI; FAJARDO, 1997), rice flour (ESCAMILLA et al., 2000), coffee husk (MACHADO et al., 2002), citrus pulp (RODRIGUES et al., 2009a), corncobs

(SATPUTE; SHARMA; MURARKAR, 2010), and jatropha seed cake (RANGASWAMY, 2012). However, only Oliveira et al. (2017), report the use of alternative substrates (citrus pulp) coupled with SSSF method for GA_3 production.

Brazil is one of the most important agriculture-based economies in the world, leading the production and exportation of agricultural products, such as coffee, sugar, ethanol, grains and orange juice. The estimation of the Brazilian harvest planted area of grains reach 58.17 million hectares, where soybean crop was responsible for 57% of the cultivated area of the country (CONAB, 2016). In this research, soybean hulls (SH) and citrus pulp (CP) were evaluated as nutrient sources to produce GA_3. SH are obtained from oil extraction processing from soybean oilseed, where for each ton of processed soybean, up to 3% of hulls are generated (KARR-LILIENTHAL et al., 2005; MONTIBELLER et al., 2014). It is estimated that soybean achieve a production and yield of around 115 million tons and 3.3 ton/ha, respectively, in 2017/2018 harvest (CONAB, 2017a).

CP is a by-product from the citrus fruit processing industry, in which rinds, seeds, and pulps are dried and pelletized, representing 50% of the fruit weigh (RODRIGUES et al., 2009b). The world production of orange in nature in 2016/17 harvest was estimated at around 49 thousand tons, where the Brazilian production was estimated at around 18 thousand tons (CONAB, 2017b).

Therefore, searches for new and low cost sources of carbon to produce GA_3 will certainly allow its large applicability, which would benefit the quality and productivity of different cultivars in a sustainable way. The objective of this research was then to produce GA_3 through SSSF using a low cost medium fermentation composed by CP and SH as alternative substrates.

3.2 MATERIAL AND METHODS

3.2.1 Microorganisms

Fusarium fujikuroi LPB 06 strain (Culture Collection of Bioprocess Engineering and Biotechnology Department of Federal University of Paraná, Brazil) was employed in this work. The strain was maintained in potato dextrose agar slants at 4ºC and sub-cultured every 3 months.

3.2.2 Substrates

Citrus pulp (CP) was obtained from Coalma Animal Nutrition - SP and soybean hulls (SH), were obtained from IMCOPA- Colombo/PR. These substrates were dried at 40 °C for 8 hours, ground in a mill, classified to a particle size smaller than 5 mm, and then stored at ambient temperature.

3.2.3 Inoculum preparation

F. fujikuroi LPB 06 was inoculated, from PDA slant, in SH or CP/SH mixtures aqueous extracts, at different ratio of 50%/50%; 70%/30% and 30%/70%, which were prepared according to the respective SSSF medium.

Inoculum was adapted from Rodrigues et al. (2009a) and Oliveira et al. (2017). An aqueous suspension with 10% (w/v) of SH or CP/SH mixtures, was incubated in a boiling water bath for 30 minutes, cooled and filtered. 50 mL of the diluted extract in deionized water (1:3) were added to a 250 mL Erlenmeyer flask. Flasks were sterilized at 121 °C for 15 minutes, inoculated with the strain and finally incubated in a rotatory shaker at 29 °C, 120 rpm, for 4 days.

3.2.4 GA_3 production in Erlenmeyer flasks

250 mL Erlenmeyer flasks were prepared with 50 mL of a suspension of 5% (w/v) of SH or a mixture with CP and SH (50%/50%; 70%/30% and 30%/70% w/w) in deionized water. The medium was sterilized at 121 °C for 15 minutes. A mycelia suspension of *F. fujikuroi* was inoculated in flasks at a rate of 10% (v/v), and incubated in a shaker at 29 °C, 120 rpm. Samples were withdrawn every 24h for GA_3 analysis.

3.2.5 GA_3 production in bioreactor

GA_3 bench scale production was carried out in a 1.5 L bubble column reactor (BCR) and 2 L stirred tank reactor (STR), in which three different medium were studied as shown in Table 3.1.

Table 3.1 – Medium composition for GA3 production in bubble column reactor (BCR) and stirred tank reactor (STR).

Medium A	**Medium B**	**Medium C**
5% (w/v) CP/SH (70%/30%)	10% (w/v) CP/SH aqueous extract (70%/30%)	ICI modified medium containing 80 g L^{-1} glucose, 1 g L^{-1} NH_4Cl, 5 g L^{-1} KH_2PO_4, 1 g L^{-1} $MgSO_4$ $7H_2O$ and 2 mL L^{-1} of a trace element solution (CANDAU; AVALOS; CERDÁ-OLMEDO, 1992; OLIVEIRA, 2012).

BCRs and STRs were sterilized at 121 °C for 20 minutes. Medium A and B were inoculated at a rate of 10% (v/v) with an inoculum prepared previously in a medium composed by CP/SH aqueous extract (70%/30%). Medium C was inoculated at a rate of 10% (v/v) with an inoculum Czapek medium that was prepared according to the manufacturer. BCRs were incubated in an acclimatized room at 29 ± 2 °C, with an aeration rate of 1 L min^{-1}. STRs fermentations were carried out using medium A and C, at 29 °C, 500 rpm and an aeration rate of 1 L min^{-1}. Samples were withdrawn every 24 hours for GA_3 analysis. SSSF was stopped at 168 hours.

3.2.6 Analytical methods

Samples were filtered to remove biomass and CP and SH particles. Sample clarification step was carried out using the Carrez solution described by LU et al. (1995) where 50 mL were prepared in volumetric flasks with 30 mL of fermented extract, 2 mL of zinc acetate (30% w/v), 2 mL of potassium ferrocyanide (15% w/v), 10 mL of ethanol and the volume was completed with distilled water. The solution was stirred vigorously and then filtered through paper filter. Quantitative determination of GA_3 concentration was performed by spectrophotometry method with acidification of clarified fermented extract or GA_3 standard solution with HCl (30% v/v) for 60 minutes at 20°C. Absorbance was determined at 254 nm (HOLBROOK; EDGE; BAILEY, 1961). Reducing sugars analysis was carried out by Somogyi-Nelson method (NELSON, 1944; SOMOGYI, 1945) and pH was determined by potentiometric method. CP and

SH composition were evaluated using Flash 2000, CHNS-O analyzer (Thermo Scientific – USA).

3.2.7 Medium cost analysis

The cost of the CP/SH (70%/30%) GA_3 production medium was compared to the cost of CP medium studied by Oliveira et al. (2017), and with the ICI synthetic medium through a simple sensibility analysis. It was calculated from the price variations found for each component (Table 3.2), in which, it was possible to analyze an optimistic and a pessimistic prices scenario. Sterilization of the medium and other costs, such as transport, labor, equipment purchase, maintenance, and operation, were not considered for this analysis. Prices obtained in Brazilian Reais were converted to dollars based on an average price quotation made in the last 12 months (US$ 1,00 = R$ 3,23).

Table 3.2 - Price variations for each medium component.

	Components	**Concentration (g L^{-1})**	**Price variations (US$/ton)**		
			Min	*Med* [c]	*Max*
Medium A (CP/SH 70%/30%)	Citrus pulp[a]	35	60	125	190
	Soybean hulls[a]	15	90	155	220
Medium B (Oliveira et al. 2017)	Citrus pulp[a]	50	60	125	190
	Sucrose[b]	20	80	340	600
	Urea[b]	0.6	100	350	600
Medium C (ICI)	Sucrose[b]	80	80	340	600
	NH_4NO_3[b]	1	200	480	760
	KH_2PO_4[b]	5	700	2500	4300
	$MgSO_4.7H_2O$[b]	1	50	425	800
	$FeSO_4.7H_2O$[b]	1	50	135	220
	$CuSO_4.5H_2O$[b]	0.15	1000	2000	3000
	$ZnSO_4.7H_2O$[b]	1.61	400	950	1500
	$MnSO_4.4H_2O$[b]	0.1	300	900	1500
	$(NH_4)_6Mo_7O_{24}$ $4H_2O$[b]	0.1	1000	23000	45000

[a]Prices available in: http://www.mfrural.com.br
[b]Prices available in: https://www.alibaba.com
[c]Calculated from min. and max.

3.2.8 Statistical analysis

Tests were carried out in triplicate to ensure reproducibility and data was submitted to analysis of variance (ANOVA) followed by Duncan's test, at 95% of confidence level ($p \leq 0.05$). Statistical analyses were performed using the software Statistica Version 7.0 (Minneapolis, USA).

3.3 RESULTS AND DISCUSSION

3.3.1 Determination of citrus pulp and soybean hulls ratio for GA_3 production through semi-solid fermentation

This study focused on the definition of the best CP and SH ratio for fermentation medium composition to GA_3 production through SSSF. CP was previously employed by Rodrigues et al. (2009a), who optimized GA_3 production in SSF. Moreover, Oliveira et al. (2017) used the same subproduct for GA_3 production via SmF and SSSF. Therefore, this study follows the research developed by the cited authors, using CP supplemented with SH as fermentation medium aiming the improvement of GA_3 production via SSSF.

The elementary composition of CP and SH is described in Table 2. It is possible to observe that CP showed higher C/N ratio than SHs, 40.98 and 20.78, respectively. Their mixtures were proposed to balance the C/N ratio and supplement the medium with an N-rich source. C/N is an important factor that affects GA_3 production. Machado et al. (2002) achieved a GA_3 production of 230.5 mg kg^{-1} using a C:N ratio of 7.3. Moreover, C:N ratios between 10:1 and 25:1, even reaching 200:1 are commonly used in SmF for GA_3 production (BORROW et al., 1964; KUMAR; LONSANE, 1990; TUDZYNSKI, 1999; SHUKLA; CHAND; SRIVASTAVA, 2005b; RODRIGUES et al., 2012b).

Table 3.3 - Elementary composition and C:N ratio of citrus pulp (CP), soybean hulls (SH), and their mixtures.

	Composition (%)					C:N ratio
	C	N	H	S	O	
CP	39.35	0.96	7.22	1.45	51.02	40.98
SH	40.12	1.93	7.60	0.176	50.17	20.78
CP/SH (30/70%)	39.88	1.63	7.49	0.56	50.44	24.46
CP/SH (50/50%)	39.73	1.44	7.41	0.808	50.61	27.59
CP/SH (70/30%)	39.59	1.25	7.33	1.06	50.77	31.67

Therefore, CP was studied in a mixture with SH for GA_3 production, where with only SH (61.62 mg L^{-1}, productivity of 0.51 mg $L^{-1}h^{-1}$) the production of the plant hormone was lower than with the different mixtures of CP/SH (Table 3.4). An improvement in GA_3 production was achieved using the CP/SH (70%/30%) mixture (451.42 mg L^{-1}) in 120 hours (3.76 mg $L^{-1}h^{-1}$), which corresponds to a C:N ratio of 31.67.

Table 3.4 - GA_3 production in Erlenmeyer flasks, with citrus pulp (CP) and soybean hulls (SH) in suspensions, after 120h of fermentation.

Conditions	**GA_3 (mg L^{-1})**	**GA_3 productivity (mg $L^{-1}h^{-1}$)**
SH	61.62 ± 8.84 (d)	0.51 ± 0.07 (d)
CP/SH (30/70%)	433.10 ± 4.41 (b)	3.60 ± 0.037 (b)
CP/SH (50/50%)	407.60 ± 11.38 (c)	3.40 ± 0.09 (c)
CP/ SH (70/30%)	451.42 ± 10.80 (a)	3.76 ± 0.09 (a)

Different letter in the same column are significant different at $p<0.05$ by Duncan's test

SH presents lower C:N ratio (20.78) and low content of fermentable sugars, which can explain the low GA_3 production. SH contain around 29 ~ 51% of cellulose (mainly glucose), 10 ~ 20 % of hemicellulose (mannose and xylose), 1 ~ 4% of lignin, and 6 ~ 15% of pectin (mainly arabinose, galactose and rhamnose) (MIELENZ; BARDSLEY; WYMAN, 2009; YOO et al., 2011). This fact points out for the necessity of chemical or enzymatic pretreatments for sugars liberation before fermentation. However, the mixtures of SH with CP, which have more availability of fermentable sugars (20% of total sugars) (OLIVEIRA et al., 2017) and higher C:N ratios, favored GA_3 production.

Oliveira et al. (2017) supplemented GA_3 fermentation medium with sucrose and urea. In this case, the mixture of these substrates, especially in the proportion 70%/30% resulted in higher C:N ratio (31.67), and higher GA_3 production. Moreover, SH can be used as nitrogen source intead of urea, which is more expensive. It is important to consider that no carbon and nitrogen chemical sources have been added to fermentation medium. From these results, the work was then carried out with inoculum and fermentation medium composed by CP and SH mixtures at 70% and 30%, respectively.

GA_3 production and reducing sugars consumption was evaluated in a time course and showed in Figure 3.1. It is possible to observe a growing GA_3 production in the first 72 hours and then there was a stabilization, where GA_3 production reached

maximum production (397 mg L^{-1}) after 168 hours of fermentation (2.36 mg $L^{-1}h^{-1}$). Reducing sugars were practically exhausted in the first 72 hours. According to Rios-iribe et al. (2011) the fungus uses only glucose for its growth in a respiratory chain. In the end of this period, the secondary metabolism, which is responsible for gibberellins production begins.

Their synthesis is also related to the presence of nitrogen in the medium. Authors report that GA_3 production starts only when the nitrogen of the medium is exhausted, which occurs after 24 to 96 hours of fermentation (ESCAMILLA et al., 2000; SHUKLA; CHAND; SRIVASTAVA, 2007; RIOS-IRIBE et al., 2011). Nitrogen content was not evaluated, although, since GA_3 production started in the first 24 hours, it is presumed that N content was already exhausted. The pH oscillated between 5.1 ~ 8.0 (parameter not controlled during fermentation). Escamilla et al. (2000) reported that pH is an important parameter that affects GA_3 production, and pH control during fermentation can improve its production.

A similar kinetics behavior was obtained by Rios-iribe et al. (2011) who evaluated GA_3 production and glucose uptake. They reached around 144 mg L^{-1} at 240 h and glucose exhaustion occurred after 144 h of fermentation. The authors also reported that gibberellins synthesis are not associated with microbial growth. Higher GA_3 production being was achieved after 170 h when glucose and nitrogen source were exhausted, and maximum biomass was reached.

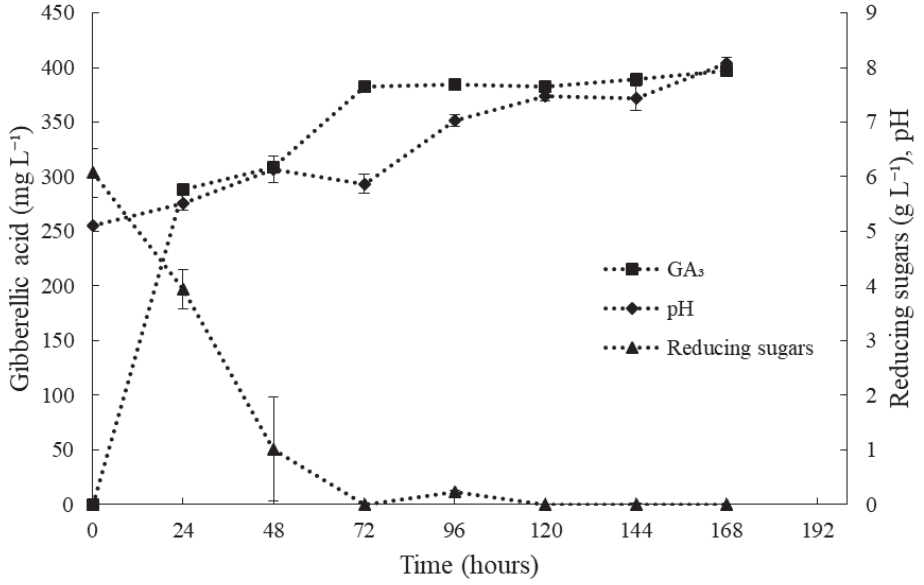


Figure 3.1 - GA_3 production, reducing sugars consumption and pH during fermentation by SSSF in Erlenmeyer flasks.

A gain of 36% was reached in GA_3 production when the mixture CP and SH (70%/30%) was employed (451 mg L^{-1} or 9.02 g kg^{-1} of CP/SH), when compared with the results obtained by Oliveira et al. (2017) using only CP (331 mg L^{-1}, 7.70 g kg^{-1} dry CP) with a C:N ratio of 40.98. This fact shows that it is important to find the ideal C:N ratio that favors the plant hormone accumulation. Kumar and Lonsane (1990) and Agosin et al. (1997), in SSF, obtained 1.1 g kg^{-1} and 6.8 g kg^{-1} of GA_3 using wheat bran, respectively. Machado et al. (2002) produced 492 mg kg^{-1} of GA_3 in coffee husk, and Rodrigues et al. (2009a) reached 5.8 g kg^{-1} of GA_3 with CP. The SSSF technique, with alternative substrates, is very promising. The advantages of using SSSF instead of SSF is mainly related to the increase of nutrients availability, the easy scaling-up of the process, and the control of fermentation parameters, which is easier than in SSF (SELVARAJ; MURTY, 2017).

3.3.2 GA_3 production in BCR

GA_3 production was carried out by SSSF and SmF in BCR. *F. fujikuroi* LBP 06 was cultivated in SSSF with CP/SH (70%/30%) in suspension, in SmF with CP/SH aqueous extract (70%/30%) and in SmF with the synthetic ICI medium. GA_3 production was evaluated and it is presented in Figure 3.2.

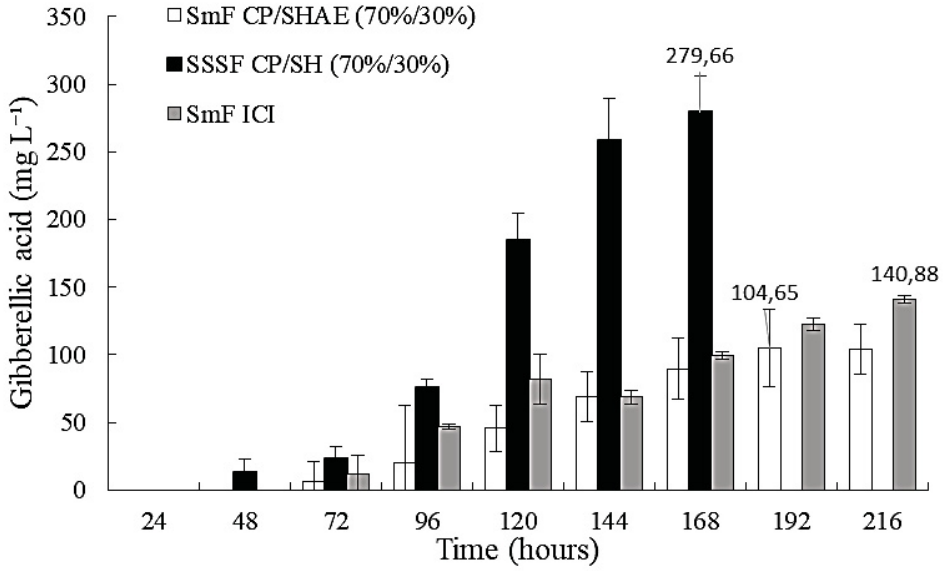


Figure 3.2 - GA_3 production in BCR using SSSF with CP/SH in suspension, SmF with CP/SH aqueous extract (CP/SHAE) and SmF with ICI synthetic medium.

According to the results, SSSF with CP/SH in suspension promoted higher GA_3 production, comparing to the SmF in both tested medium (CP/SHAE and ICI medium). The presence of solid substrates in suspension seems to promoted a support effect for the fungus' growth, and consequently improving GA_3 production. Maximum GA_3 production reached 279.66 mg L^{-1}, with a productivity of 1.66 mg L^{-1} h^{-1}. Lower GA_3 productivities were obtained with ICI medium and CP/SH aqueous extract, 0.65 mg L^{-1} h^{-1} and 0.54 mg L^{-1} h^{-1}, respectively.

These results are lower than those achieved in Erlenmeyer flasks. This fact is probably due to the forced aeration that affected the fungus development at the employed rate or the aeration did not provide medium oxygenation and agitation. It was observed a deposition of the particulated material in the bottom of the bioreactor, showing an inneficient homogenization and, consequently, an insufficient oxygen transfer. GA_3 biosynthesis involves many oxidative steps, which are catalyzed by cytochrome P450 monooxygenases, dioxygenases and dehydrogenases though, a good aeration of bioreactor is critical for an optimal production process (TUDZYNSKI, 1999). Furthermore, the thickening broth tends to decrease oxygen transfer, and the resulting oxygen restriction drastically reduces GA_3 formation (VASS; JEFFERYS, 1979;TUDZYNSKI, 1999).

There are few reports related to GA_3 production using BCR, most of the bioreactor studies are focused on STRs (RIOS-IRIBE et al., 2011; RIOS-IRIBE;

HERNÁNDEZ-CALDERÓN; ESCAMILLA-SILVA, 2016) and airlift reactors (CHAVEZ-PARGA et al., 2006, 2008). Only Oliveira et al. (2017) worked with BCR and SSSF for GA_3 production achieving a productivity of 0.94 mg L^{-1} h^{-1} using CP aqueous extract and 0.96 mg L^{-1} h^{-1} using CP in suspensions.

The knowledge of hydrodynamics and mass transfer parameters in BCRs is necessary for a proper scale-up of the process. The evaluation of the regime flow, bubble size distribution, and coalescence characteristics, gas holdup, interfacial mass transfer coefficients, gas-liquid interfacial area, dispersion coefficients and heat transfer coefficients is recommended for fermentation process behavior and optimization (CHAVEZ-PARGA et al., 2006, 2008). No information was found about BCR parameters studies for GA_3 production, thus these study will contribute to its production scale-up.

3.3.3 GA_3 production in STR

GA_3 production was carried out in STR in SSSF and SmF. *F. fujikuroi* LBP 06 was cultivated in SSSF with CP/SH (70%/30%) in suspension and in SmF with ICI medium in a 2 L STR. GA_3 production was evaluated and it is presented in Figure 3.3.

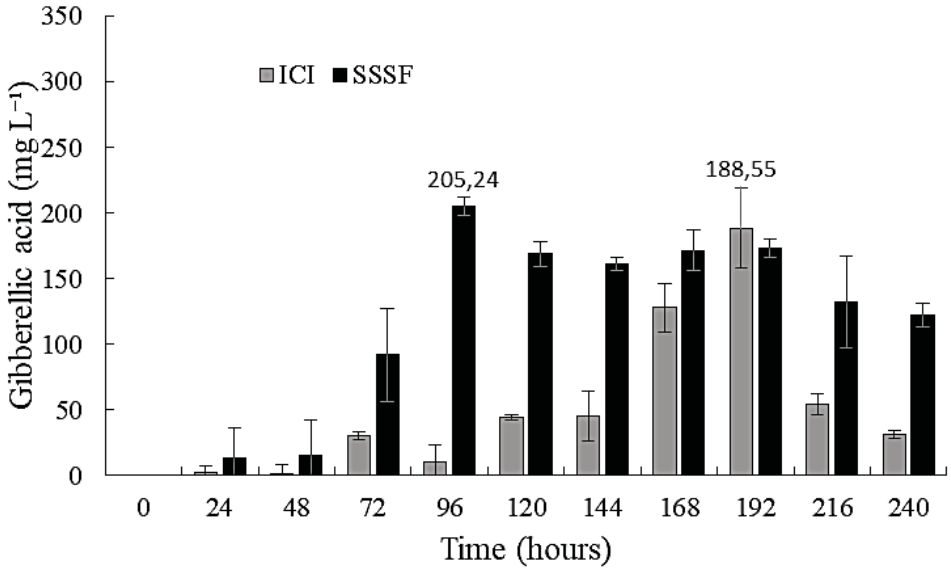


Figure 3.3 - GA_3 production in STR using SSSF with CP/SH in suspension, and SmF with ICI synthetic medium.

GA_3 production in SSSF (205.24 mg L^{-1}) with CP/SH showed better results than using ICI medium (188.55 mg L^{-1}) with 2.20-fold productivity (2.14 mg L^{-1} h^{-1}). For both tested medium higher GA_3 productivities were obtained in STR then in BCR. Therefore, STR promoted better medium homogenization and oxygenation, improving process's yield.

Rios-iribe et al. (2011) used a Continuous Stirrer Tank Reactor (CSTR) and reached a productivity of 1.31 mg L^{-1} h^{-1} using glucose and corn oil as substrates, and 0.51 mg $L^{-1}h^{-1}$ using only glucose. Oliveira et al. (2017) obtained a productivity of 2.84 mg $L^{-1}h^{-1}$ using an aqueous extract of CP. The authors did not test the GA_3 production through SSSF in STR system. Therefore, this work is the first report about GA_3 production via SSSF in STR.

3.3.4 Sensibility analysis of GA_3 medium cost

The cost of GA_3 fermentation medium was estimated through a sensibility analysis in optimistic (empty column) and pessimistic scenarios (full column) (Figure 3.4). Figure 3.4a, describes the price variation for each component, according to the concentration used, for the medium used in this work (CP/SH), in which CP showed the greatest influence on the price (84.5%), ranging from US$ 4,43/m^3 to US$ 8,98/m^3. In the medium adopted by Oliveira et al. (2017) (Figure 3.4b) the added sucrose showed significant influence on medium price (71.9%), which oscillated between US$ 8,00/m^3 to US$ 18,46/m^3. The price of the synthetic medium ICI (Figure 3.4c) varied from US$ 24,00/m^3 to US$ 66,00/m^3, in which 81.64% of the price was mainly influenced by sucrose.

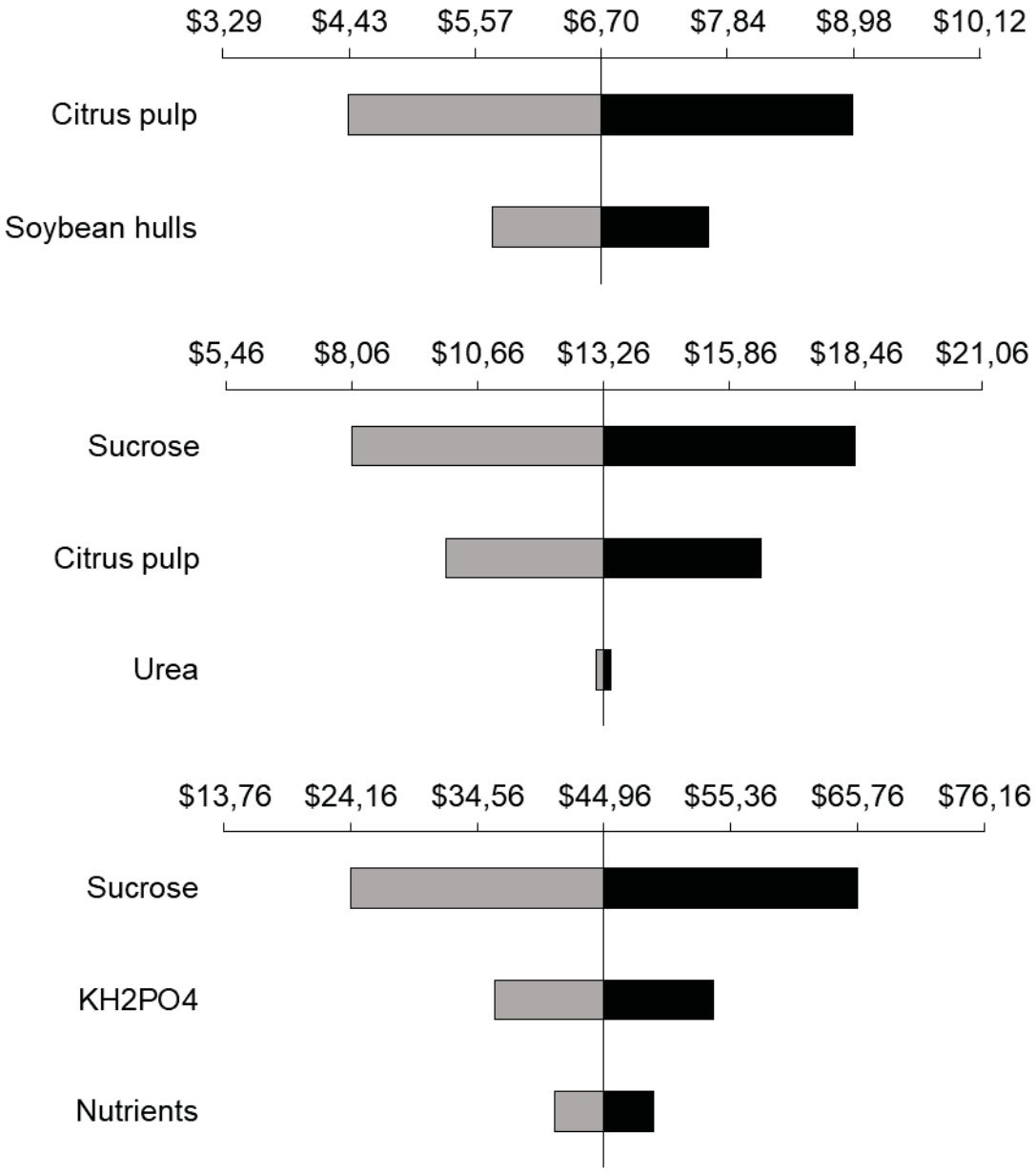


Figure 3.4 – The cost of each component (US$/m^3) in an optimistic (empty column) and pessimistic (full column) scenarios for mediums used in this work (CP/SH) (A), used by Oliveira et al. (2017) (B), and a synthetic medium (C).

For all compared fermentation medium, the carbon source, which is also used in greater quantity, was the component that most influenced prices. Even so, the average price of the culture medium A is estimated as US$ 6,70 / m³, while the prices of medium B and medium C go around US$ 13,26 / m³ and US$ 44,96 / m³, respectively (Figure 3.5). Medium A reduced the prices of the culture medium in 49.4% compared with medium B and 85% when compared with medium C. Therefore, based on the results obtained in BCR (see 3.3.2) the average of GA_3 production cost is expected to

be US$ 23,92/kg of GA_3 using CP/SH (70%/30%) and US$ 279,25/kg of GA_3 using ICI. Nevertheless, the use of CP and CS as fermentation medium for GA_3 production is a very good strategy in the search of economic solution to enable to plant growth hormone's large application for different cultivars.

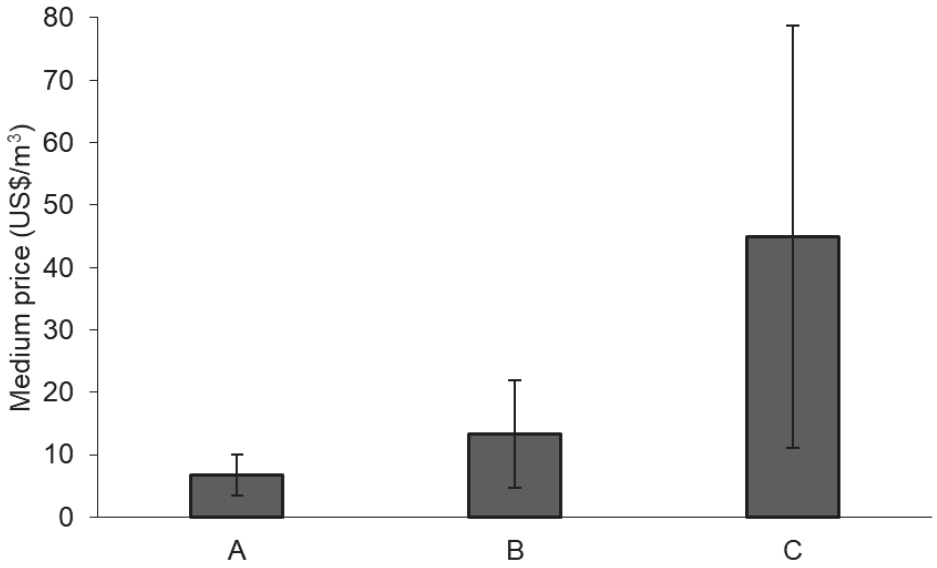


Figure 3.5 - Estimation of culture medium prices in US$/m³. A – medium CP/SH (70%/30%); B – medium reported by Oliveira et al. (2017); C – ICI.

3.4 CONCLUSIONS

A simple alternative bioprocess for GA_3 production was proposed. It involves the use of citrus pulp (CP) and soybean hulls (SH) as substrate in semi-solid fermentation. The production of GA_3 by *Fusarium fujikuroi* LPB 06 reached 451.42 mg L^{-1} (9.02 g kg^{-1} of CP/SH) in 120 hours of fermentation, corresponding to a productivity of 3.76 mg L^{-1} h^{-1} in Erlenmeyer flasks, with a ratio 70%/30% of CP/SH. The scale up of GA_3 production was carried out in bubble column reactor (BCR - 1.5 L) and in stirred tank reactor (STR – 2 L). Higher GA_3 productivities were obtained in STR, but lower than in Erlenmeyer flaks. Thus, studies of fermentation parameters in both reactors are necessary to improve GA_3 production by SSSF. Even so, higher productivities were obtained with the use of alternative complex medium than with synthetic medium for both tested reactors (2.55-fold and 2.20-fold higher in BCR and STR, respectively). CP and SH are cheap and widely generated in Brazil, served as a support for fungus

growth, and reduced in 85% the price of the culture medium, becoming a new economically option for this bioprocess.

CHAPTER 4 - PREPARATION OF STABLE LIQUID AND POWDER GIBBERELLIC ACID FORMULATIONS

Marcela Candido Camara[a]; Luciana Porto de Souza Vandenberghe[a*]; Valcineide O A Tanobe[a]; Natascha Amalio Teixeira[a]; Cristine Rodrigues[a] and Carlos Ricardo Soccol[a]

[a] Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, Brazil, Centro Politécnico, Curitiba-PR, 81531-980, Phone number: 005541 33613271

*Corresponding author: lvandenberghe@ufpr.br

ABSTRACT

Gibberellic acid (GA_3) is a high value plant growth hormone commonly used to improve the quality of agricultural products. The development of a stable liquid and powder GA_3 formulation was studied, which was produced by semi-solid fermentation using 70% of citrus pulp and 30% of soybean hulls. The fermented extract was purified through ultrafiltration membranes. For liquid fermentation, adjuvants such as preservatives (PA and PB), and surfactants (SA, SB and SC) were tested. Diluents (DX, DY, DZ and DW) were also evaluated. Preservatives and surfactants that were added to GA_3 purified extract formulation, promoted some protective effect on its activity. DX (50% and 90%) and DY (90%) showed a better effect on GA_3's stability maintaining 62%, 63% and 76%, respectively, of its activity at accelerated conditions (54°C during 14 days). A final formulation of the GA_3 clarified extract was defined with DX (50%) and SA (2.5%), which promoted a conservation of 66% of the molecule's stability after 14 days at 54°C and 99% after 6 months in ambient conditions storage. GA_3 powder formulation was obtained by spray drying. It was studied the effects of carrier A (CA) (10, 15 and 20%) and inlet temperature (120°, 150° and 180°C) in the quality of dried powder and GA_3 stability. GA_3 powder formulation was defined with 10% of CA, dried at 180°C, showing a powder stability around 90% after 8 months. GA_3 stable and simple formulations were developed and therefore being potential products for application in a variety of commercially important cultivars.

Keywords: Plant growth regulator. Semi-solid fermentation. GA_3. Liquid formulation. Powder formulation. Spray dryer.

4.1 INTRODUCTION

Gibberellic acid (GA_3) is a natural plant growth regulator (PGR) belonging to the gibberellins (GA) family. Its commercial importance is linked to properties related to plant development, such as seed germination, abiotic stress tolerance, and plant and fruit growth (ORTEGA-BAES AND ROJAS-ARÉCHIGA, 2007; CASANOVA et al., 2009; AKTER et al., 2014; ZANG et al., 2016).

The fungi *Fusarium fujikuroi* is the main source of GA_3 in industry (SHUKLA; CHAND; SRIVASTAVA, 2005a). Unfortunately, its production usually presents low yields making GA_3 a high added-value molecule, which restricts its use. It has been reported that GA_3's annual production is around 100 tons at a price of around $25/g (RANGASWAMY, 2012; ALBERMANN et al., 2013).

The majority of studies involving GA_3 are concerned with its production, and so there are few reports focused on downstream processes. Its recovery can be carried out through liquid-liquid extraction (ESCAMILLA et al., 2000; USLU, 2012; USLU; DATTA; BAMUFLEH, 2014) or adsorption (TANG et al., 2000; WANG et al., 2008; RODRIGUES et al., 2016). Active charcoal and Carrez solution are employed for fermented broth clarification (LU; XIE; KUMAKURA, 1995; MELEIGY; KHALAF, 2009; RODRIGUES et al., 2016). Membrane separation methods can be used for organic acids recovery (MAGALHÃES et al., 2017). However, no reports were found related to GA_3 separation through diafiltration or eletrodialysis, even with ultrafiltration techniques.

GA_3 is currently commercialized in liquid, powder, granules or tablet formulations. Liquid formulations generally comprises alcoholic solutions, because GA_3 is hydrolyzed in water (HOLLMANN AND GEIPEL, 1995; DEVISETTY et al., 2007). Ethanol, isopropyl alcohol, methyl alcohol, propanol, and butanol are commonly used as solvents. In addition, acetone, ethyl lactate, butyl lactate, or low volatile compounds such as polyethylene glycol, glycerol or propylene glycol are also reported (DATTA et al., 2006; WANG et al., 2013; DEVISETTY et al., 2015).

Soluble powder formulations showed some advantages in comparison to liquid formulations, such as higher GA_3 stability, smaller packaging and easier transportation. In this type of product, the active ingredient is combined with a finely ground dry carrier, as examples, sorbitol, mannitol, lactose, dextrose, starch, limestone, mineral clay, talc,

sucrose, maltose, maltodextrin, and fructose. There are different methods that have been used to obtain powder formulations, among then extrusion and spray drying are commonly used. Devisetty et al. (2007) reported GA_3 drying by extrusion, however, no reports were found about GA_3 drying in spray dryers. This drying technique is the oldest and well-established method to formulate huge quantities of material in a minimal continuous processing operation as well as it is a low cost technology and easiness of industrialization (ANANDHARAMAKRISHNAN; RIELLY; STAPLEY, 2008; KARTHIK; ANANDHARAMAKRISHNAN, 2013).

These formulations can be supplemented with additives and/or adjuvants to provide additional characteristics to the formulated product. Moreover, they can also act as protectors increasing the stability and shelf life of the product (DEVISETTY et al., 2006, 2015; PAWLAK, 2016).

Some patents report GA_3 or GA_4 and GA_7 products. In the US 3038794 patent, authors claim GA_3 products in powder, liquid, spray and paste form (GEARY; BEACH; HABER, 1962). Liquid formulations using polyethylene glycol and surfactants were claimed by Devisetty et al. (2015) and Wang et al. (2013). Soluble granules obtained with a mixture of lactose, a surfactant and a binder was claimed by Devisetty et al. (2006). However, little is known about GA_3 liquid and powder formulation processes and about its shelf life studies in current literature.

The largest producers are international companies, such as Valent BioSciences Corp., Syngenta Crop Protection and the Australian Nufarm®. Furthermore, China and India are also important suppliers of GA_3. The development of Brazilian techniques for the production of GA_3, aiming the reduction of production and importation costs, would therefore promote a larger and continuous application of GA_3, since Brazil has one of the most important agriculture-based economies in the world.

In this work, the ultrafiltration technique was employed to separate and purify the GA_3 produced by a simple technique of semi-solid fermentation. Liquid GA_3 formulation study was carried out by testing different additives and solvents. Finally, the spray drying method was used to obtain a GA_3 powdered product and test the addition of adjuvants and under different drying conditions.

4.2 MATERIAL AND METHODS

The scheme of the fermented extract formulation process is shown in Figure 4.1.

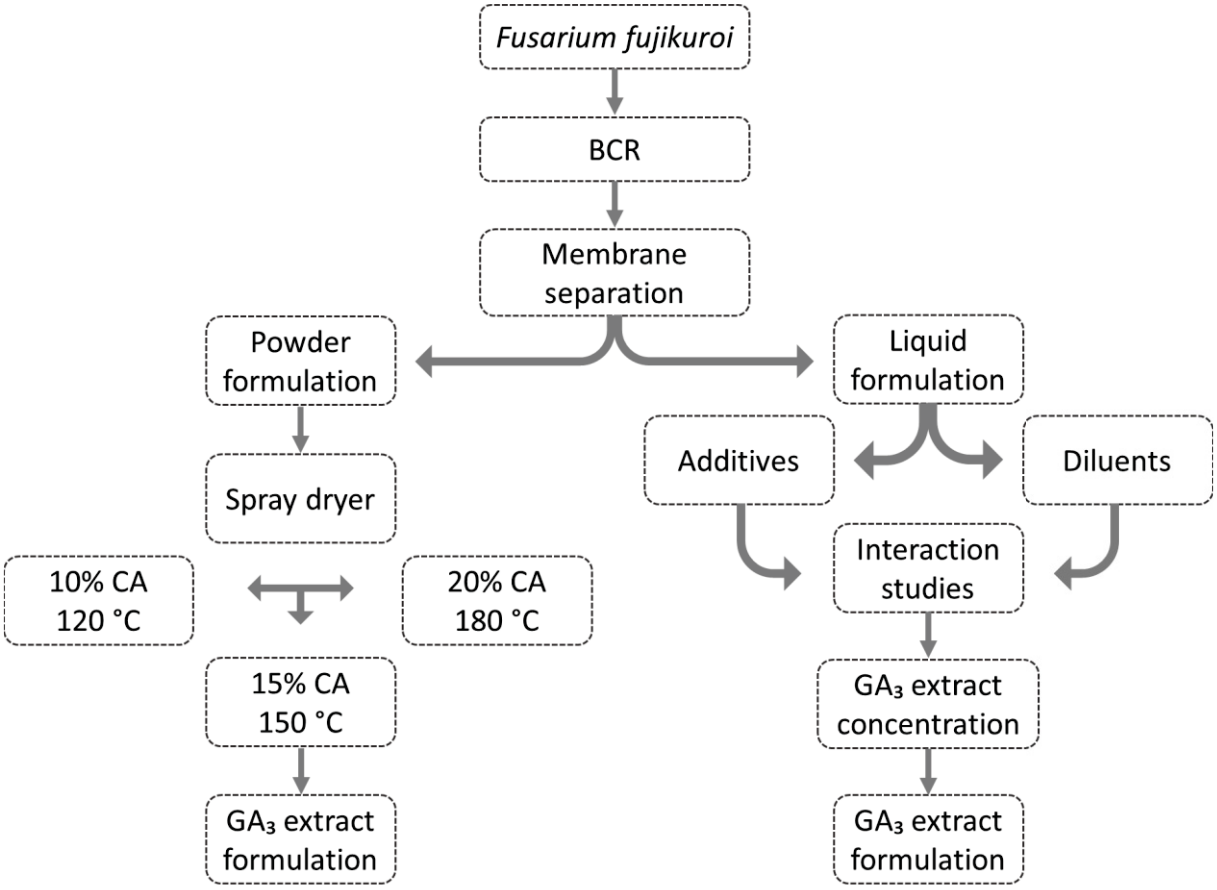


Figure 4.1 – Process of GA_3 extract powder and liquid formulation.
Source: author

4.2.1 Microorganisms

Fusarium fujikuroi LPB 06 from the strain bank of Bioprocess Engineering and Biotechnology Department of Federal University of Paraná, was employed in this work. The strain was grown in PDA (Potato Dextrose Agar) slants at 29 ºC for 7 days, stored at 4 ºC and subcultured every 3 months.

4.2.2 Substrates

The alternative substrates, citrus pulp (CP), which was obtained from Coalma Animal Nutrition- SP and soybean hulls (SH), from IMCOPA - Colombo/PR, were employed in GA_3 production. These substrates were dried at 40 °C for 8 hours, ground in a mill, classified to a particle size smaller than 5 mm, and stored.

4.2.3 Inoculum preparation

F. fujikuroi LPB 06 was inoculated, from PDA slant, in the medium composed of CP/SH mixture aqueous extract. Inoculum was prepared according to Rodrigues et al. (2009) and Oliveira et al. (2017). An aqueous suspension with 10% (w/v) of CP/SH mixture (70%/30% w/w), was incubated in a boiling water bath for 30 minutes in glass flasks, cooled and filtered. 50 mL of the diluted extract in deionized water (1:3) were added to a 250 mL Erlenmeyer flask. Flasks were sterilized at 121 °C for 15 minutes, inoculated and incubated in a shaker at 29 °C, 120 rpm, for 4 days.

4.2.4 GA_3 production by semi-solid fermentation in bubble column reactor (BCR)

Semi-solid fermentation was carried out in a 1.5 L BCR with CP/SH medium. BCR was prepared with 1 L of deionized water and a suspension of 5% (w/v) of CP/SH mixture in the proportion of 70%:30% w/w. BCR was sterilized at 121 °C for 20 minutes. Medium was inoculated at a rate of 10% (v/v) from previously prepared inoculum medium. BCR was incubated in an acclimatized room at 29 ± 2 °C, 1 vvm of aeration, for 5 days.

4.2.5 GA_3 extract purification

GA_3 crude extract was purified though microfiltration (MF), with 0.22 µm, and ultrafiltration (UF) with 10 kDa and 100 kDa using a Pellicon® (Merk Millipore, Germany) tangential flow filtration system. Before experiments, membranes were rinsed by pumping ultrapure water, and then stored with a solution of 0.1N NaOH solution, according to the manufacturer. After use, membranes were cleaned by recirculating 0.1N NaOH for 30-40 minutes, according to the manufacturer's instructions.

4.2.6 GA_3 liquid formulation

The pre-selection of the formulation ingredients and their tested concentrations for the GA_3 formulation were based on previous works, and according to their functional activity (DEVISETTY et al., 2006; WANG et al., 2013). Preservatives increases the shelf life of a product by preventing or delaying changes caused by microorganisms. Surfactants act as wetting or spreading agents and their main role is to act on the surface of a droplet to reduce the surface tension. This allows the droplet to spread over the leaf surface (CASTRO; OJEDA; CIRELLI, 2014). Therefore, ingredients belonging to both classes were selected for screening studies. Their names were codified due to a patent process that is occurring.

First, GA_3 purified extract was formulated and evaluated with adjuvants. Two groups were studied, preservatives and surfactants. The first with 2.5% (w/v) PA or PB, and the second with 2.5% (w/v), SA, SB or SC. Samples were maintained at 54 °C for 14 days and GA_3 stability was evaluated.

DX, DY, DZ and DW were tested as diluent. 0.1% GA_3 standard (Fluka) solution was prepared with 10%, 50% or 90% (v/v) of each diluent. Samples were maintained at 54 °C for 14 days (MARTIN; DOBRAT, 1994) and GA_3 activity was evaluated.

4.2.6.1 *Experimental design*

Full factorial design (2^3) was used to evaluate the effects of the interaction between DX (25 – 75 %), SA (0 – 5 %) and PB (0 – 5 %), on GA_3 activity. Eleven experiments and three replicates at the center point, were carried out with 0.1% of standard GA_3 (Fluka) solution. Coded and uncoded values are described in Table 4.1. Samples were maintained at 54 °C for 14 days (MARTIN; DOBRAT, 1994) and GA_3 concentration was evaluated.

Table 4.1 - Coded and uncoded values adopted in the experimental design to study the influence of factors diluent X (DX), surfactant A (SA) and surfactante B (PB) on GA_3 stability.

Coded values	Factors		
	DX (% v/v)	*SA (% v/v)*	*PB (% w/v)*
-1	25	0	0
0	50	2.5	2.5
1	75	5	5

4.2.6.2 GA_3 liquid formulation

GA_3 purified extract obtained after fermentation was concentrated under reduced pressure using a rotatory evaporator (*Fisatom*) at 45 °C to obtain a concentration of 47.5% from the initial volume. The concentrated extract was formulated with DX at 50% (v/v) and with the surfactant SA at 2.5% (v/v). Samples were evaluated in an accelerate stability test at 54 °C for 14 days (MARTIN; DOBRAT, 1994), and in long-term stability test at room temperature for 6 months. GA_3 activity was evaluated every two days and every month, respectively.

4.2.7 GA_3 powder formulation

An inert carrier (CA) was choose to compose powder formulation. This component is able to assist the drying process of the material and in granules formation.

The purified extract was mixed with CA at 10% (w/v), 15% (w/v) or 20% (w/v). The mixture was homogenized and immediately fed in a laboratory scale spray dryer Lab Plant SD-05 (Huddersfield, England), with a nozzle atomization system with 0.5 mm diameter. The inlet temperature ranged from 120 °C to 180 °C and the outlet air temperature were maintained at 95 ± 10 °C. Drying airflow and feed flow rate were 73 $m^3\ h^{-1}$ and 600 mL h^{-1} respectively. Compressor air pressure was fixed at 0.06 MPa (CARNEIRO et al., 2013). Experiments were performed in a full factorial design (2^2) and involved six experiments with a replicate at the center point (Table 4.2). The spray-dried powders were collected, kept in glass bottle and GA_3 activity was analyzed every month.

Table 4.2 - Coded and uncoded values adopted in the experimental design to study the effect of variables carrier A (CA) and spray dryer inlet temperature on GA_3 stability.

Coded values	Factors	
	CA (% w/v)	*Inlet temperature (°C)*
-1	10	120
0	15	150
1	20	180

4.2.8 GA_3 powder analysis

4.2.8.1 Powder yield

Powder yield was determined according to Belghith et al. (2001). The total solids (TS) and the powder yield (%) was calculated from Eq. (1) and (2), respectively:

$$TS\ (g) = (\%ES \times vol) + (\%CA \times vol) \quad (1)$$

$$Yield\ (\%) = \left(\frac{Wt}{TS}\right) \times 100 \quad (2)$$

Where: ES represents the fermented extract solid content; CA the percentage of carreador used; vol, the volume of fermented extract; and Wt, the powder weight of the samples obtained after drying.

4.2.8.2 Encapsulation efficiency

The encapsulation efficiency was determined based on the initial and final GA_3 weight difference. 0.2 g of GA_3 powder extract was diluted in 2 mL of deionized water and its activity was measured. The GA_3 concentration after drying ($[GA_3]_f$) was calculated as follow:

$$[\mathrm{GA_3}]\mathrm{f}(mg\ g^{-1}) = \frac{([GA_3] \times vol)}{TS} \quad (3)$$

Where, [GA_3] represents GA_3 concentration in mg L^{-1}; vol, the volume of fermented extract and TS total solids content in each condition. Then, GA_3 final weight (Wf(GA_3)) obtained in each studied condition was calculated according to Eq. (4):

$$Wf(GA_3)(mg) = [GA_3]f \times Wt \quad (4)$$

Where: $[GA_3]_f$ represents the GA_3 concentration after drying; and W_t, the powder weight obtained after drying. Finally, the encapsulation efficiency (EE) was calculated from Eq. (5):

$$EE(\%) = \left(\frac{Wf\ (GA_3)}{Wt_0\ (GA_3)}\right) \times 100 \quad (5)$$

Where: W_f GA_3 is the GA_3 final weight and Wt_0 is the GA_3 weight before drying process.

4.2.8.3 Moisture content

Powders' moisture content was measured gravimetrically by infrared moisture balance (Top-Ray, Bel Engineering®, Italy). The samples, approximately 3±0.5 g, were placed evenly on the balance pan. The temperature was adjusted to 105 °C and the results were shown in moisture percentage.

4.2.8.4 Scanning electron microscopy

The morphological properties of the spray-dried powders were observed in a Scanning Electron Microscope (SEM) (JEOL, JSM-6360LV). The powder samples were fixed to the SEM stubs of 12 mm diameter and then subjected to metallization with a thin layer of gold. The samples were observed with magnifications of 300 x, 750 x and 1500 x. An acceleration potential of 10 kV and backscattering electrons (BSE) mode was used during micrograph.

4.2.8.5 Particle size distribution

The average size of samples in aqueous medium was used to determined hydrodynamic diameter by DLS (Brookhaven, NanoDLS).

4.2.8.6 X-ray diffraction

The diffractogram of samples were analyzed using the X-ray diffractometer XRD-7000 (Shimadzu). Operational conditions of 40 kV and 20 mA were used. Scanning condition were 2 theta, scan range between 5 – 50 and 2.0 degree.min^{-1} scan speed.

4.2.9 Analytical methods

Quantitative determination of GA_3 concentration was performed by spectrophotometry method (UV-visible sprectrophotometer, UV-1601PC, Shimadzu-Japan) with acidification of GA_3 solutions with HCl (30% v/v) for 60 minutes at 20°C. Absorbance was determined at 254 nm (HOLBROOK; EDGE; BAILEY, 1961).

4.2.10 Statistical analysis

Tests were carried out at least in triplicate to ensure reproducibility. Statistical analyses were performed using the software Statistica Version 7.0 (Minneapolis, USA). The results were expressed according to standard deviation.

4.3 RESULTS AND DISCUSSION

4.3.1 GA_3 liquid formulation and stability studies

GA_3 extract was obtained through semi-solid fermentation with CP/SH alternative substrates and reached a concentration around 200 mg L^{-1} after 120 hours. GA_3 extract was then micro and ultrafiltrated in order to purify the extract for further steps. In this purification system, there was 73% of GA_3 recovery after microfiltration and 100 kDa and 10 kDa ultrafiltration. The GA_3 extract formulation process and its

stability was studied to ensure an adequate shelf life for this product, especially in liquid forms.

The effect of adjuvants addition on GA_3 purified extract stability was studied. Two groups of adjuvants were tested and the results are shown in Figure 4.2a and 4.2b. It is important to notice that tests were carried out under accelerated conditions, where the molecule's stability was forced to its limit of activity. In this case, GA_3 activity was tested at 54 °C up to 14 days.

The first group (Figure 4.2a) was composed by the preservatives. PA and PB increased the GA_3's stability when compared to the purified extract without these additives. GA_3's stability reached 11% and 40%, respectively, after 14 days at 54 °C. Without additives the extract lost completely GA_3's activity at the end of sotrage period.

In Figure 4.2b it is possible to observe the influence of surfactants group on GA_3 purified extract stability. SA and SC were more efficient on the preservation of the molecule's stability than SB, resulting in 24%, 25% and 15%, respectively, of GA_3 final concentration. In both cases, the purified extract without adjuvants lost the GA_3 activity completely at the end of storage period.

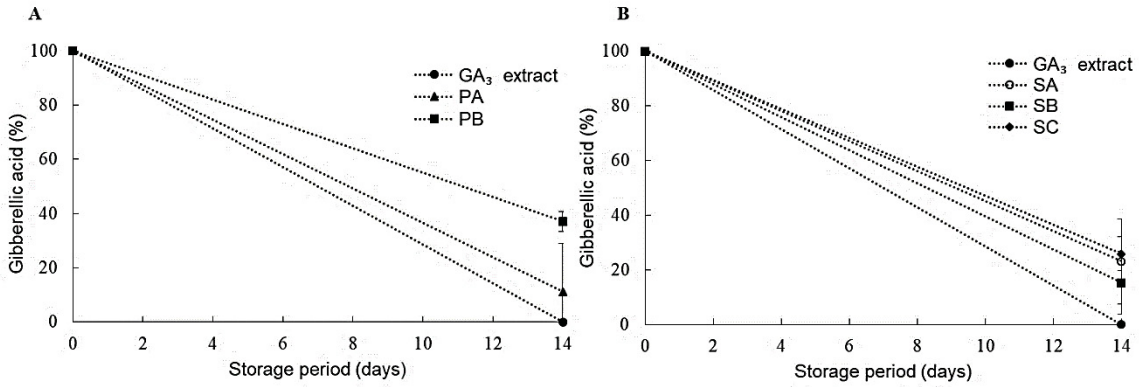


Figure 4.2 - GA_3 stability test at 54 °C with additives, (A) Preservative group: PA and PB; (B) Surfactants group: SA, SB and SC.

The lack of GA_3 stability in both cases can be associated with the presence of a double bond in its chemical structure, making the molecule more reactive. According to Albermann et al. (2013) the same occurs with GA_7, whereas GA_4 is the most stable of the three gibberellins. It has been also suggested that the loss of the lactone ring can lead to biological inactivation of gibberellins (PÉREZ et al. 1996).

All results showed a decrease in GA_3's activity. This probably occured due to the acceleration of molecule hydrolysis influenced by the high temperature used in the test. Moreover, Pérez et al. (1996) reported that the GA_3 molecule can be rapidly decomposed in aqueous solutions, and temperature, time reaction or pH also have an important role in molecule's degradation. Under these conditions, other compounds can be formed, such as gibberellenic acid, allogibberic, 9-epi-allogibberic, 9,11-didehydroallo-gibberic acid or GA_3 isomerization (iso-GA_3) in weak alkaline conditions. In these forms, the biological activity of this plant growth hormone is reduced or completely lost. Bruckner et al. (1991) related a GA_3 half-life of around 14 days at 20 °C and 2 days at 50 °C. Pérez et al. (1996) evaluating the kinetics of GA_3 decomposition in aqueous solution related half-lives of 3.2 and 2.4 days for pH 5 and 7, respectively, at 30 °C.

In the study the addition of preservatives and surfactants were not able to prevent the rapid degradation of GA_3 in aqueous solution. When water as diluent, according to previous results, it promotes the drastic loss of GA_3 stability. Commercial GA_3 liquid formulations are currently prepared with isopropyl alcohol or methyl alcohol as diluents so as to avoid the hormone hydrolysis that occurs in aqueous systems (HOLLMANN et al., 1995a; DEVISETTY et al., 2007). Thus, standard GA_3's stability was studied using different diluents, DX, DY, DZ and DW, at three concentrations (10%, 50%, 90% v/v), as shown in Figure 4.3. Low concentrations of each solution (10%) presented almost the same behavior as the control, showing that the presence of higher concentrations of water is not favorable for GA_3 stability with a fast decrease in 2 days, remaining constant for 14 days.

The stability of standard GA_3 in DX (Figure 4.3a) remained constant for 6 days at concentrations of 50% and 90%. After this period, there was a decrease of standard GA_3 stability to 60%, which was maintained up to 14 days. DZ (Figure 4.3b) was the worst diluent for GA_3 stability, leading to GA_3's final concentration of 30% with the higher DZ concentration. Contrarily, GA_3 presented good stability with DY was used as diluent (Figure 4.3c), especially at 90%. At these conditions, GA_3's stability remained constant for 6 days, with further decrease until day 8 and then stabilizing until 14 days. GA_3's final activity was 76% with DY 90%. With DY 50%, the stability was lower than with DY 90%, resulting in 40% of GA_3's activity after 14 days at 54 °C.

DW (Figure 4.3d) also presented some effect on GA_3 stability, but from the sixth day, the concentration started to increase. More studies are necessary to test the effect of this diluent and search for the causes of GA_3 activities' oscillation.

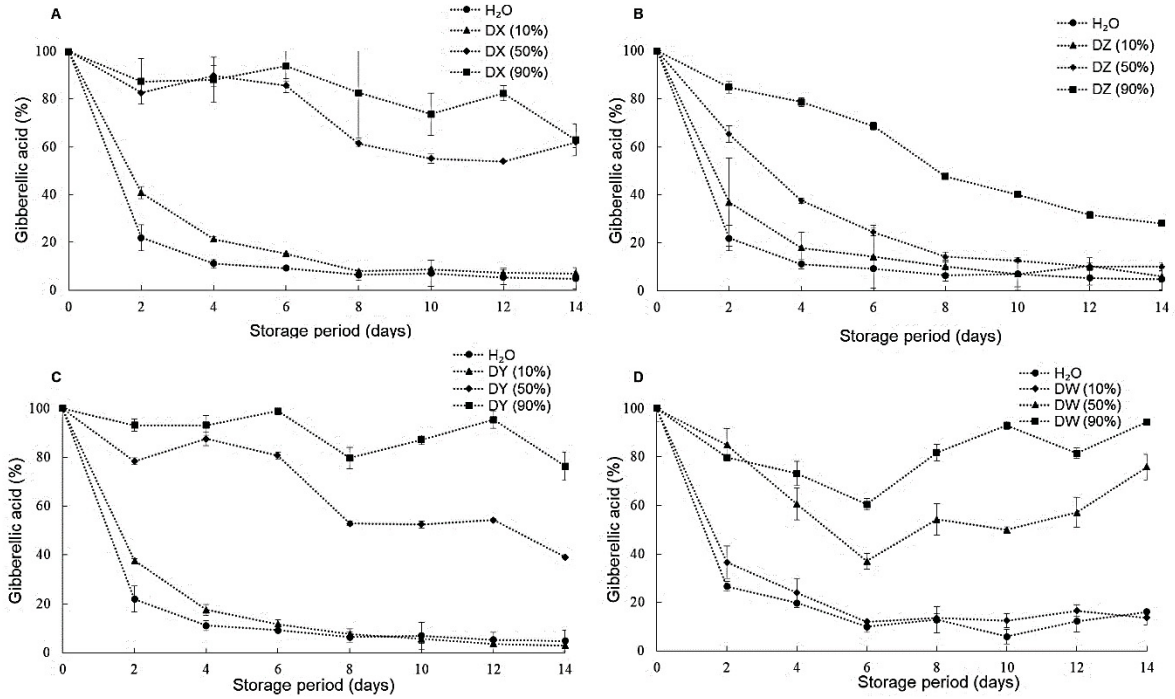


Figure 4.3 - GA_3 stability test using different diluents, (A) GA_3 diluted in 10, 50 and 90% DX; (B) GA_3 diluted in 10, 50 and 90% DZ; (C) GA_3 diluted in 10, 50 and 90% DY; (D) GA_3 diluted in 10, 50 and 90% DW.

Alcoholic solutions are commonly used aiming to ensure greater stability of the product, increasing its shelf life (DEVISETTY et al., 2007). DX was able to ensure GA_3 standard stability above 60% after exposure to the molecule's stress condition (54 °C) for 14 days. DY ensured good product stability over 14 days, however, only at high concentrations. This condition resulted in a high viscosity solution, which can lead to serious difficulties application of the product via foliar. Therefore, stability studies were continued only with DX as solvent.

4.3.2 Effect of additives interaction on GA_3's stability

DX was evaluated in interaction with PB and SA. The adjuvants were chosen due to their effect on GA_3 purified extract stability. The test was carried out in a 2^3 full factorial design experiment. The presence of DX was significant, and the more concentrated the more it showed its protective effect on GA_3 stability. PB showed a

negative effect on GA_3 molecule, and in high concentration, crystals were observed. The addition of SA did not influence GA_3 stability (Table 4.3).

Table 4.3 - Effects of DX, SA and PB on GA_3 stability after 14 days at 54 °C.

Assay			**GA_3 stability (%)**
DX (%)	*SA (%)*	*PB (%)*	
25	-	-	22.64
75	-	-	97.91
25	5	-	10.08
75	5	-	95.49
25	-	5	48.20
75	-	5	55.77
25	5	5	41.79
75	5	5	58.03
50	2.5	2.5	47.03
50	2.5	2.5	41.70
50	2.5	2.5	42.99

Surfactants are adjuvants that are responsible for improving agrochemicals' performance. They are able to increase the foliar uptake by breaking the surface tension of the water, and causing a uniform spreading, exposing the spray droplets on a larger foliar surface and, consequently, increasing the absorption. According to Castro et al. (2014) agrochemical formulations require the use of surfactant not only for maintenance of long-term physical stability, but also for enhancing the biological performance of the agrochemical, particularly for herbicides, growth regulators and defoliants.

The use of surfactants for foliar application with growth regulators have been reported by Pérez-jiménez et al. (2016). Likewise, to ensure penetration into leaf tissues, Rady and Mohamed (2015) reports the addition of surfactants for foliar application of *Moringa oleifera* leaf extract and salicylic acid in bean plants. Thus, based on the importance of the addition of surfactant to ensure a better absorption of growth regulators by plants, the addition of SA, in the lowest concentration (2.5%), to the formulated was maintained.

4.3.3 GA_3 extract formulation

The purified extract obtained after fermentation contains water diluted GA_3. Therefore, to obtain a stable product from this extract, it is necessary a concentration step and then a formulation with the solvent that could allow better GA_3 stability. In this case, it was used DX combined with the surfactant SA.

Although in the previous tests higher DX concentrations proved to be better for maintaining the GA_3 stability, DX at 50% with addition of SA was used to formulate the GA_3 purified extract. This DX concentration was chosen to avoid toxicity in plants during application. The GA_3 purified extract and the formulated extract stability showed similar behavior when subjected to high temperatures, resulting around 67% of GA_3 activity after 14 days (Figure 4.4). The GA_3 formulated extract also showed similar behavior compared to GA_3 standard formulated with DX at 50% (Figure 4.3a).

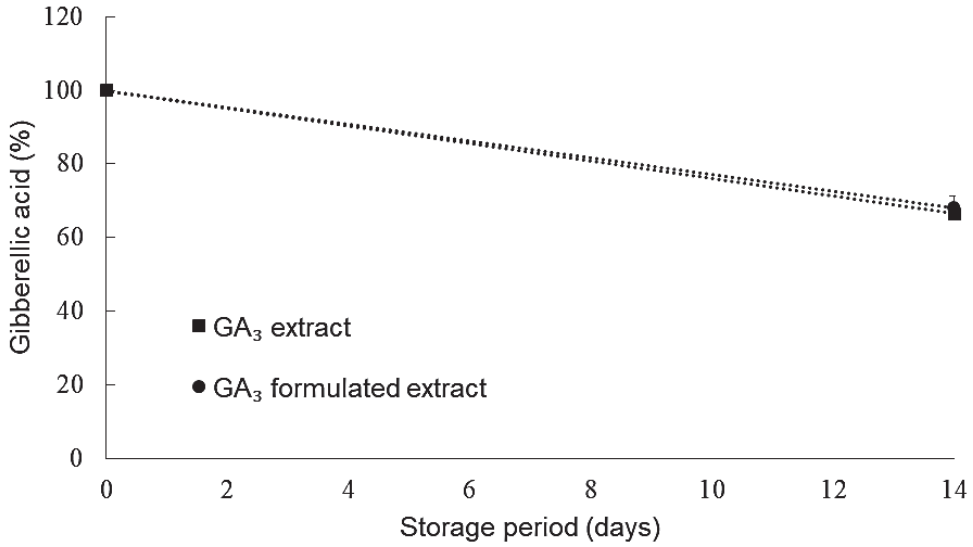


Figure 4.4 - Stability test of GA_3 purified extract and GA_3 purified extract formulated with diluent X (DX) (50%) + surfactant A (SA) (2.5%) at 54 °C for 14 days.

However, at room temperature, GA_3 formulated extract was significantly more stable than the non-formulated extract, maintaining 99% and 45% of GA_3 stability after 6 months, respectively (Figure 4.5). These results are in agreement with the literature, where it is possible to observe the low GA_3 stability in aqueous solutions (HOLLMANN; GEIPEL, 1995; PÉREZ et al., 1996; DEVISETTY et al., 2007).

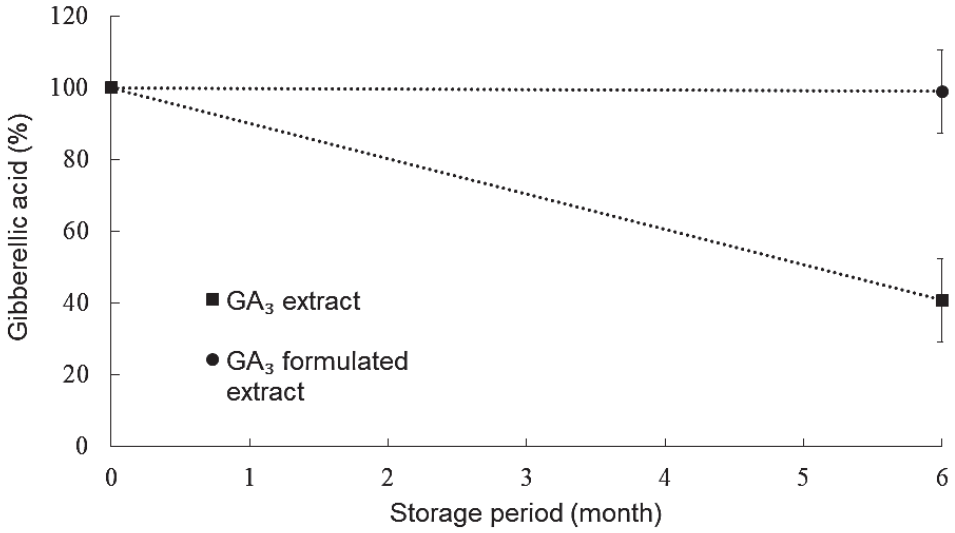


Figure 4.5 - Stability test of GA_3 purified extract and GA_3 purified extract formulated with diluent X (DX) (50 %) + surfactant A (SA) (2.5 %) at room temperature for 6 months.

The non-formulated extract stored at room temperature showed some contamination, which may have contributed to the loss of GA_3 activity. According to Hollmann et al. (1995), GA_3 can be metabolized by microorganisms in the absence of carbon sources.

The accelerated storage test surely exposed the active biomolecule to its maximum stress. The objective is to simulate the normal long-term ageing of a formulation by heating. In this test, the loss of the active ingredient up to 5%, guarantees a shelf life of two years or more. In the case of high sensitive active ingredients, a long-term stability test or alternative conditions should be carried out to evaluate the product shelf life (MARTIN; DOBRAT, 1994; FAO, 2010). Due to the GA_3 susceptibility to temperature, the short-term test was not efficient in determining the shelf life of this product. Thus, the stability of the product was evaluated in a long-term test, and as seen, the formulated product remained practically stable over a period of 6 months. This indicates that the developed product can be marketed and safely applied, ensuring a shelf life of 6 months or more.

4.3.4 GA_3 powder formulation

4.3.4.1 Process efficiency

Powder formulation of GA_3 purified extract was obtained using the spray dryer technique. CA was used as wall material, due to its functional properties, such as relatively low cost, low viscosity at high concentrations and good protection against oxidation (CARNEIRO et al., 2013). Spray dryer process yield, encapsulation efficiency and powder moisture are shown in Table 4.4. The process presented low yield, however, the condition B (10% CA at 180 °C) showed the highest process yield (21.60%) and encapsulation efficiency (21.19%). The results also suggested that higher CA concentrations were not able to enhance the GA_3 encapsulation efficiency.

Table 4.4 - Process yield, encapsulation efficiency and moisture content of spray dried GA_3 powders.

Assay	CA (%)	Inlet temperature (°C)	Powder yield (%)	Encapsulation efficiency (%)	Moisture content (%)
A	10	120	7.59	8.57	0.09
B	10	180	21.60	21.19	0.06
C	15	150	5.59	7.70	0.05
D	15	150	16.79	20.83	0.06
E	20	120	1.03	0.85	0.16
F	20	180	4.49	2.54	0.07

In general, the type of wall material used may affect the encapsulation efficiency. The low encapsulation efficiency achieved here could be explained due to CA low emulsifying capacity or even the design and operational conditions of the used equipment (spray dryer). According to Carneiro et al. (2013), low emulsifying capacity is the CA biggest problem and it can affect the efficiency of microencapsulation in spray dryers. In order to avoid this problem, it is desirable to use it in combination with others surface-active biopolymers. The same authors evaluated a combination of CA with four other types of wall material for microencapsulation of flaxseed oil. The best encapsulation efficiency was reached using CA in combination with modified starch (95.7%).

Loksuwan (2007), comparing the β-carotene microencapsulation with modified and native tapioca starch, and CA, showed that the lowest total carotene content in microcapsules was obtained with CA (46.74%) when compared with modified (82.18%) and native (68.35%) tapioca starch. The author also correlated the low β-carotene retention to the CA lack of emulsification and low film-forming capacity.

An unexpected result was obtained when higher CA concentration was used. Tonon et al. (2011) observed that the increase in the total of solids' content resulted in higher oil encapsulation efficiency. Balasubramani et al. (2015) and Rajabi et al. (2015) also reported similar results. Such results can be correlated with the increase in the emulsion viscosity, reducing the circulation movements inside the droplets and, leading to a rapid skin formation (JAFARI et al., 2008; TONON; GROSSO; HUBINGER, 2011). Therefore, CA concentration used for GA_3 extract encapsulation may not provide an ideal viscosity, affecting the encapsulation process. Thus, new studies seeking to optimize the emulsion viscosity of the sample may result in an improvement of the process, reducing the GA_3 losses.

4.3.4.2 GA_3 powder morphology

The influence of different concentrations of CA and inlet spray drying temperatures on microcapsules' structure was observed by SEM. Results (Figure 4.6) show that the inlet temperature interferes on powder dry quality and consequently, on particle morphology. Microcapsules agglomeration was observed using the inlet temperature of 120 °C (Figure 4.6a and Figure 4.6e). Particles showed less uniform shape and smooth surface with some roughness particles. These characteristics can be resulted due to the relatively high water content in the particle wall material. In fact, as it can be seen in Table 4.4, with an inlet temperature of 120 °C, higher powder moisture content was obtained, suggesting that lower temperatures did not provide an efficient powder drying. Similar condition was observed by Wang et al. (2011).

When inlet temperature was increased to 150 °C, it is possible to observe differences in microcapsules morphology (Figure 4.6c and Figure 4.6d). Irregular shaped with extensive dented or roughness surface microcapsules were obtained. The same occurred using the condition with 20% CA and 180 °C (Figure 4.6f). In Figure 4.6b, using 10% CA and 180 °C, the particles showed spherical shape and some roughness surface. Roughness surface is explained by rapid particle shrinkage during the drying process. Similar morphology was observed in β-carotene encapsulation (LOKSUWAN, 2007), oils encapsulation (WANG; TIAN; CHEN, 2011; CARNEIRO et al., 2013) and gallic acid encapsulation (MEDINA-TORRES et al., 2013).

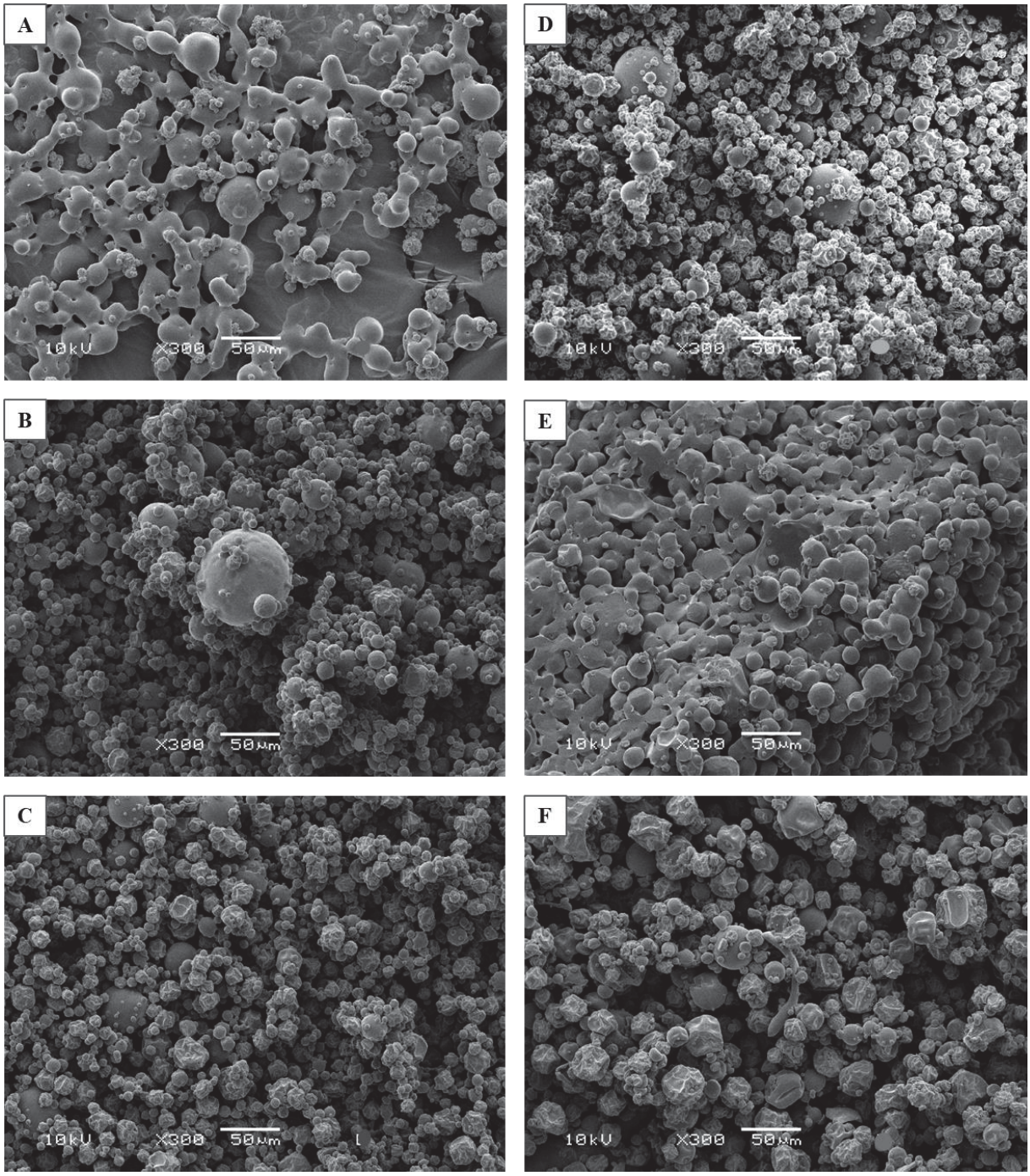


Figure 4.6 - Micrographs of GA_3 purified extract powder microcapsules obtained by SEM. (A) GA_3 extract with 10 % carrier A (CA) and inlet temperature of 120°C, (B) GA_3 extract with 10 % CA and 180°C, (C) and (D) GA_3 extract with 15 % CA and 150°C, (E) GA_3 extract with 20 % CA and 120°C, and (F) GA_3 extract with 20 % CA and 180°C (magnification of 300 – 10 kV).

4.3.4.3 Particle characterization of GA_3 powder

The average of particle size is shown in Figure 4.7. The analysis was carried out with the spray drying condition B (10% CA and inlet temperature of 180 °C), which

showed the best powder yield and encapsulation efficiency. Particle size ranged from 2-20 µm, where around 60 % of the particles were classified at 10-15 µm. The variety in sizes is a typical characteristic of particles produced by spray drying (CARNEIRO et al., 2013; RAJABI et al., 2015).

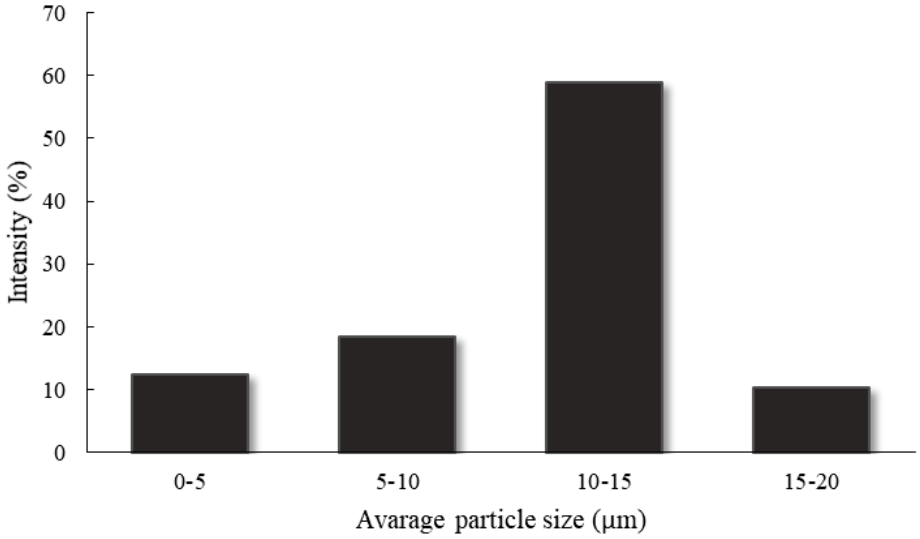


Figure 4.7 - Particle size distribution of GA_3 purified extract powder formulated with 10% carrier A (CA) and dried at 180 °C.

In Figure 4.8 it is described the powder X-ray diffraction. According to the results, GA_3 extract powder showed an amorphous characteristic due to the presence of a non-defined and large peak. This behavior was similar to those described by Cano-chauca et al. (2005) evaluating the induction of crystallization in mango powders. Amorphous powder is obtained from the rapid drying of the feed liquid droplets, resulting in a powder with sticky properties. These properties may lead to wall deposition that affects the process yield (CHIOU; LANGRISH, 2007).

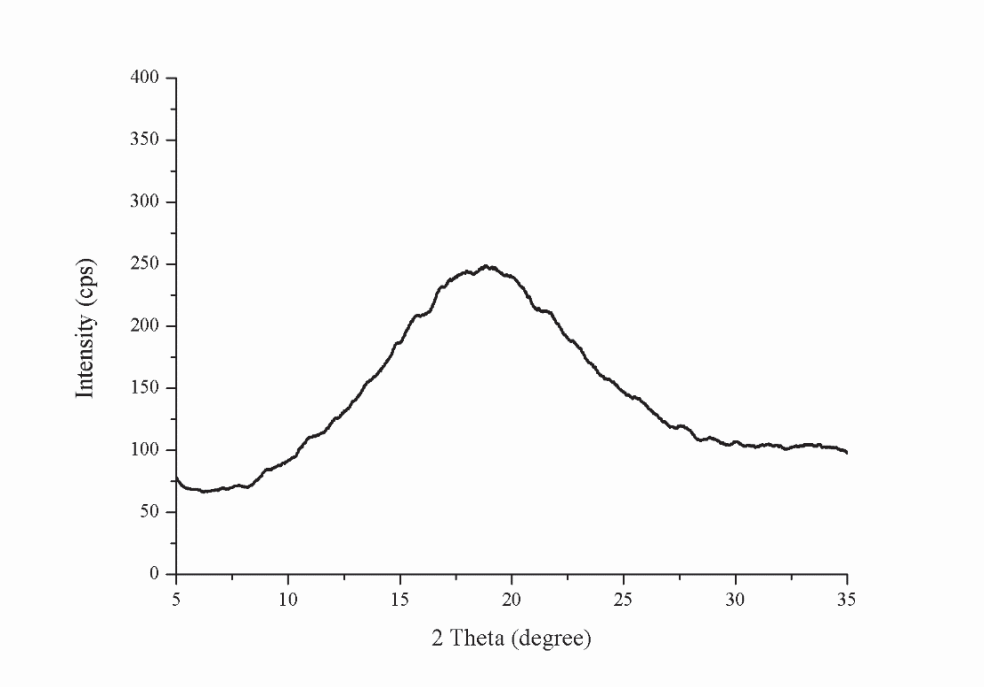


Figure 4.8 - X-ray diffraction of GA_3 extract powder formulated with 10% carrier A (CA) and dried at 180 °C.

Furthermore, due to the hygroscopic nature of the obtained powder, colloidal silica dioxide, an anti-caking and anti-humectant agent were evaluated in order to avoid sticky properties. However, no differences were found when compared to the condition with only addition of CA (data not shown). Therefore, due to those hygroscopic and sticky characteristics of GA_3 extract powder, to ensure its stability, it is important to store the product in sealed package free of moisture.

4.3.4.4 GA_3 powder stability

GA_3 extract powder stability was evaluated and the results are shown in Figure 4.7. The powder product obtained by spray dryer, remained practically stable over 8 months, reaching around 90% of GA_3 activity. The powder formulation is more advantageous than liquid formulation to prevent GA_3 hydrolysis. Moreover, it requires smaller size of packaging, lower transport costs and storage space (DEVISETTY et al., 2007).

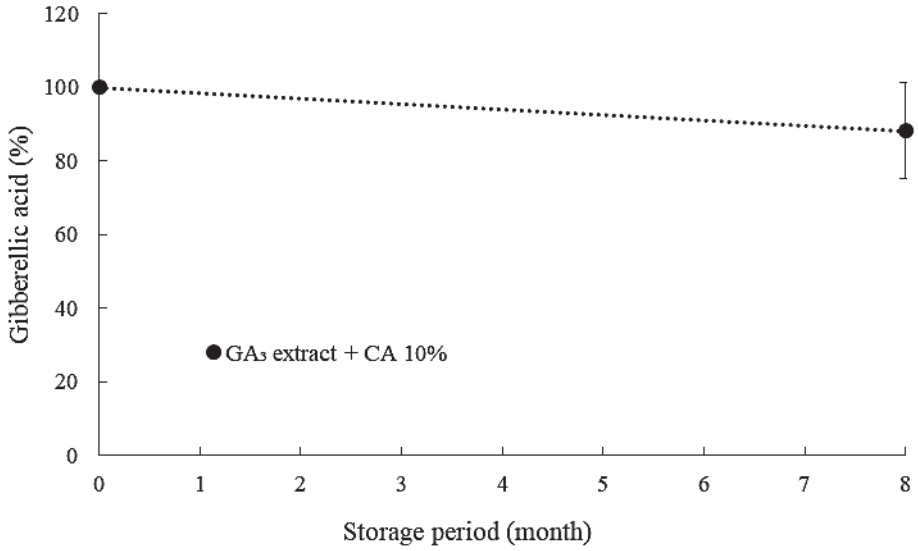


Figure 4.9- Stability test of GA_3 powder extract formulated with 10% carrier A (CA) and dried at 180 °C at room temperature for 8 months.

Despite the low yield obtained during the drying process of GA_3 formulated extract, it can be observed that the spray dryer technique was effective in obtaining a high value-added stable product. However, the conduction of new studies related to the optimization of the drying process will contribute to a better performance and project design of the equipment obtaining a high quality GA_3 product, which can be applied in several types of crops and fruits.

4.4 CONCLUSIONS

This research focused on the development of liquid and powder formulations containing a commercially important plant growth hormone. GA_3 was obtained through semi-solid fermentation and semi-purified using micro and ultrafiltration method. A stable liquid formulation was developed using 50% of DX, as diluent, and 2.5% of SA, as formulation adjuvant. In this condition, the GA_3 activity remained almost constant, while the GA_3 extract without formulation lost 55% of its activity after 6 months of storage. The powder product was obtained through spray dryer, in which the GA_3 purified extract, was formulated with different concentrations of CA. The best drying condition was reached with GA_3 extract added of 10% of CA. GA_3's powder activity remained around 90% after 8 months. The development of simple, stable, safe and low cost GA_3 formulations will certainly allow its wide application in agriculture cultivars

such as ornamental flowers, crop plants and fruits, increasing its quality and production.

CHAPTER 5 - DEVELOPMENT OF GIBBERELLIC ACID (GA_3) PREPARATIONS AND THE EFFECTS OF EXOGENOUS APPLICATION IN SOYBEAN

Marcela C Camara[1]; Luciana P S Vandenberghe[1*]; Tales Romano[2]; Oranys I M Gonzalez[1]; João Carlos Bespalhok Filho[2]; Carlos R Soccol[1]

[1]Bioprocess Engineering and Biotechnology Department, Federal University of Paraná, Brazil, Centro Politécnico, CP 19011, Curitiba-PR, 81531-908, Phone number: 005541 33613697

[2]Department of Plant Science and Crop Protection, Federal University of Paraná, Brazil, CP 2959, Curitiba, PR, 80035-050.

*Corresponding author, e-mail: lvandenberghe@ufpr.br, 005541 33613697

ABSTRACT

Gibberellic acid (GA_3) is a plant growth regulator commonly used for crop stimulation and stress tolerance. The current study evaluated the production of liquid and powder GA_3 formulations, and the effects of its exogenous application in soybean. GA_3 was produced by semi-solid fermentation using citrus pulp and soybean hulls (70%/30%) in 1.5L bubble column reactor (BCR). Fermented extract was purified through micro and ultrafiltration membranes system and formulated. In liquid solution, GA_3 extract was formulated with 50% of DX and 2.5% of SA, named Gibbtec LS. In powder formulation, GA_3 extract was formulated with 10% of CA and lyophilized, it was named Gibbtec SP. Six treatments were employed to evaluate seed germination, T1- without immersion; T2- Water; T3- Progibb® 400; T4- GA_3 extract; T5- Gibbtec LS; and T6- Gibbtec SP. T6 showed the lowest mean germination time (MGT) (3.08 days), followed by T3 and T4 (3.41 and 3.45 days). T6 achieved highest germination percentage (96%), which was 15 % more than T3. Considering root length and lateral root formation, T4 and T6 promoted the best results. GA_3 pre-treated seeds (T3, T4 and T6) induced shoot elongation when compared to treatments T1 and T2. Foliar applications were used to evaluate GA_3 products on plant development. Three applications were made in plant stages V3, R1 and R4, with the treatments: T1- water; T2- Progibb® 400; T3- GA_3 extract; T4- Gibbtec LS; and T5- Gibbtec SP. The foliar applications in plant development showed that treatments containing GA_3 significantly increased plant stem elongation when compared to control (T1). T2 presented the highest plant height and shoot dry matter (143.8 cm and 12.42 g), followed by T3 (113.33 cm and 11.14 g) and T5 (105.03 cm and 10.23 g). T1 presented 61.60 cm of plant height and shoot dry matter of 9.86 g. Treatments with GA_3 did not influence root dry matter or soybean yield. However, it is possible to say that the developed products have great potential to be used in the agriculture for seed treatment and plant growth stimulation.

Keywords: GA_3. Liquid formulation. Powder formulation. *Glycine max*. Progibb® 400. Semi-solid state fermentation.

5.1 INTRODUCTION

The development of new technologies for crop improvement, such as higher yield, environmental stress tolerance, insects/disease resistance and higher quality of grains has been the focus of researches for years. The use of plant growth hormones, specially gibberellic acid (GA_3), to stimulate the development of seeds, shoots, root, flower and fruits have been extensively reported (MACLEOD; MILLAR, 1962; BALAGUERA-LOPEZ; CARDENAS-HERNANDEZ; ALVAREZ-HERRERA, 2009; KOYUNCU, 2005; ATAY; KOYUNCU, 2016; ZANG et al., 2016).

GA_3 can be applied on seeds or via foliar. The role of GA_3 in seeds treatment is to improve germination by the induction and secretion of hydrolytic enzymes in the aleurone layer, releasing nutrients for the growing embryo (HEDDEN; SPONSEL, 2015; RADEMACHER, 2015). Moreover, treated seeds are able to withstand the adverse effects caused by high salinity soils (NASRI et al., 2012), and overcome dormancy of recalcitrant seeds (KOYUNCU, 2005).

Foliar applications usually provide to plants resistance to saline or drought stress and improve the development of stem and fruit due to the GA_3 property on promoting cell elongation and cell division. In maize plants, GA_3 foliar application alleviated stresses caused by drought (AKTER et al., 2014) and salt excess (TUNA et al., 2008). Exogenous application in soybean and sugarcane also alleviate the toxic effects of salt stress (HAMAYUN et al., 2010; SHOMEILI et al., 2011). CASANOVA et al. (2009) and ZANG et al. (2016) reported fruit quality improvement when GA_3 was applied in grapes and blueberry, respectively. Despite the properties of GA_3 related to cultivar stimulation, it is not very widespread due to its relatively high costs, which may not be economically viable to the producer.

GA_3 is industrially produced by submerged fermentation (SmF) through *Fusarium fujikuroi*. However, new methods for its production looking for cost reduction and high productivity have been studied (ESCAMILLA et al., 2000; RIOS-IRIBE et al., 2011; RIOS-IRIBE; HERNÁNDEZ-CALDERÓN; ESCAMILLA-SILVA, 2016). Nowadays, different GA_3 products are commercially available and most of them comprises dried formulations. It is the most commercialized plant growth regulator and its global use is around 100 tons per year, in which China is the biggest seller

(RADEMACHER, 2015). Nowadays, GA_3 price for agricultural application ranges from US$ 150/kg to US$ 500/kg (prices searched in alibaba.com, accessed in 04/2018).

GA_3 can be applied in many cultures including cereal grains til fruits. Valent BioSciencesCorp., a leading developer and provider of GAs, describe more than 40 different plant species, in which their GA preparations can be utilized. Even though Brazil has its economy based on agriculture, there is still scarce application of exogenous sources of plant growth promoters, such as GA_3, which may be a promising market for the product because of its potential and the diversity of cultures in which it can be used.

Nevertheless, the development of new competitive GA_3 products reducing its production cost it is necessary and it will allow its widespread application. Therefore, we proposed two preparations containing GA_3, produced by semi-solid fermentation and the evaluation of its biologic activity on seed germination and seedling growth comparing to the GA_3 commercial product.

5.2 MATERIAL AND METHODS

5.2.1 Microorganism

Fusarium fujikuroi LPB 06 from the strain bank of Bioprocess Engineering and Biotechnology Department of Federal University of Paraná was employed in this work. The strain was grown in PDA (Potato Dextrose Agar) slants at 29 ºC for 7 days, stored at 4 ºC and subcultured every 3 months.

5.2.2 Substrate

The alternative substrates, citrus pulp (CP), which was obtained from Coalma Animal Nutrition- SP and soybean hulls (SH), from IMCOPA- Colombo/PR, were employed in GA_3 production. These substrates were dried at 40 °C for 8 hours, ground in a mill, classified to a particle size smaller than 5 mm, and stored.

5.2.3 Inoculum preparation

F. fujikuroi LPB 06 was inoculated, from PDA slant, in the medium composed of CP/SH mixture aqueous extract. Inoculum was adapted from Rodrigues et al. (2009) and Oliveira et al. (2017). An aqueous suspension with 10% (w/v) of CP/SH mixture (70%/30% w/w), was incubated in a boiling water bath for 30 minutes in glass flasks, cooled and filtered. 50 mL of the diluted extract in deionized water (1:3) were added to 250 mL Erlenmeyer flasks. Flasks were sterilized at 121 °C for 15 minutes, inoculated and incubated in a shaker at 29 °C, 120 rpm, for 4 days.

5.2.4 GA_3 production by semi-solid state fermentation in bubble column reactor (BCR)

Semi-solid fermentation was carried out in a 1.5L BCR with CP/SH medium. BCR was prepared with 1L of deionized water and a suspension of 5% (w/v) of CP/SH mixture in the proportion of 70%/30% w/w. BCR was sterilized at 121°C for 20 minutes. Medium was inoculated at a rate of 10 % (v/v) from previously prepared inoculum medium. BCR was incubated in an acclimatized room at 29 ± 2 °C, 1 vvm of aeration, for 5 days.

5.2.5 GA_3 extract purification

GA_3 crude extract was filtrated to remove biomass and CP/SH particles. Sample clarification step was carried out using the Carrez solution described by Lu et al. (1995) in which 50 mL volumetric flasks were prepared with, 30 mL of fermented extract, 2 mL of zinc acetate (30% w/v), 2 mL of potassium ferrocyanide (15% w/v), 10 mL of ethanol and the volume was completed with deionized water. The solution was stirred vigorously and then filtered.

In an alternative process, GA_3 crude extract was filtrated and purified though microfiltration (MF), with 0.22 µm, and ultrafiltration (UF) with 10 kDa and 100 kDa using a Pellicon® (Merk Millipore, Germany) tangential flow filtration system. Before experiments, membranes were rinsed by pumping ultrapure water. After use, membranes were cleaned by recirculating 0.1N NaOH for 30-40 minutes, and then stored with the same solution, according to the manufacturer's instructions.

5.2.6 GA_3 liquid formulation

GA_3 extract obtained after purification steps was concentrated under reduced pressure using a rotatory evaporator (*Fisatom*) at 45 °C so as to obtain a concentration of 47.5% from the initial volume. The concentrated extract was formulated with diluent X (DX) at 50% (v/v) and with the surfactant A (SA) at 2.5% (v/v). The liquid formulation of GA_3 purified extract was named as Gibbtec LS (liquid solution).

5.2.7 GA_3 powder formulation

To the purified extract was added of carrier A (CA) at 10% (w/v). The mixture was homogenized and then, lyophilized (RVT4104 - Thermo Scientific), at – 45 °C for 24 h, in an alternative dry process in order to overcome the lower yields promoted by spray dryer process, evaluated in Chapter 4. The powder formulation of GA_3 extract was named as Gibbtec SP (soluble powder).

5.2.8 Bioactivity assays

Soybean (*Glycine max* L., Pampeana 20RR cultivar) were supplied from Department of Bioprocess and Biotechnology Engineering, Federal University of Paraná. Progibb® 400 SG (Valent BioSciences – Sumimoto, São Paulo) was used as GA_3 commercial product.

5.2.8.1 Germination assays

The soybean seeds were treated by immersion for 5 min in different solutions. The assay comprised six treatments: T1- without immersion; T2- deionized water; T3- ProGibb® 400 SG; T4- GA_3 purified extract; T5- Gibbtec LS; and T6- Gibbtec SP. The GA_3 concentration was 100 mg L^{-1}. The seeds were sown in germination trays filled with vermiculite, maintained in greenhouse at 29 ± 2 °C and irrigated when necessary and 100 seeds were used per treatment.

The effects of GA_3 solutions on seed germination were evaluated using the variables: germination percentage, mean germination time, length of the primary root,

number of lateral roots and shoot length. The percentage of germination was calculated using:

$$\% \text{ Germination} = \frac{X \,.\, 100}{Y} \quad (1)$$

Where X is the number of germinated seeds and Y is the total number of seeds used in the assay.

Mean germination time (MGT) (days) was calculated using:

$$MGT = \frac{\sum n.t}{\sum n} \quad (2)$$

Where n is the number of newly germinated seeds at time t, and t is the time from the beginning of the germination test in days.

5.2.8.2 Plant development

To evaluate the effect of GA_3 on plant development, seeds of soybean were sown in 1 L capacity pots, which were filled with vermiculite and kept in a greenhouse. Foliar application of GA_3 products was carried out in soybean seedlings. First application occurred in vegetative stage 3 (V3) (Figure 5.3a), when the plant presents three nodes on the main stem with the presence of fully developed leaves. The second application occurred in the reproductive stage 1 (R1) (Figure 5.3b), when the plant has at least one open flower at any node of the main stem, and in R4 (Figure 5.3c), when there are pods with 2 cm long at one of the four uppermost nodes of the main stem with fully developed leaf (ZHANG; PAN; SMITH, 1997).

The following treatments were employed: T1- deionized water; T2- ProGibb® 400 SG; T3- GA_3 purified extract; T4- Gibbtec LS; and T5- Gibbtec SP. GA_3 concentration was kept in 100 mg L^{-1} for all treatments. Hoagland's nutrient solution containing 1 mM KH_2PO_4, 5 mM KNO_3, 5 mM $Ca(NO_3)_2$ $5H_2O$, 2 mM $MgSO_4$ $7H_2O$, 1 mM Fe-EDTA, and 1 mM of micronutrients was used as the fertilizer once a week (HOAGLAND; ARNON, 1950). Plants were irrigated with 100 mL of deionized water

when necessary. The assay consisted of twenty pots per treatment, and each pot contained two plants.

Figure 5.1 – Different development stages of soybean in which the application occur. A - V3 stage, first application; B - R1 stage, floral development and second application; C - R4 stage, developed pod and third application.

After harvesting, the following variables were analyzed: plant height (cm), aerial dry matter mass (g), root dry mass (g), number of pods per plant and number of grains per plant. The dry mass (g) was determined by oven drying the samples at 60°C for two days.

5.2.9 Analytical methods

Quantitative determination of GA_3 concentration was performed by spectrophotometry method (UV-visible spectrophotometer, UV-1601PC, Shimadzu-Japan) with acidification of GA_3 solutions with HCl (30% v/v) for 60 minutes at 20 °C. Absorbance was determined at 254 nm (HOLBROOK; EDGE; BAILEY, 1961).

5.2.10 Statistical analysis

Data was submitted to analysis of variance (ANOVA) followed by Duncan's test, at 95% of confidence level ($p \leq 0.05$). Statistical analyses were performed using the software Statistica Version 7.0 (Minneapolis, USA).

5.3 RESULTS AND DISCUSSION

5.3.1 Seed germination

Two types of GA_3 formulations consisting of a liquid solution (LS) and a soluble powder (SP), which were produced from a fermented extract, were evaluated. The GA_3 extract passed through two purification methods involving a clarification by precipitation and membrane purification by micro and ultrafiltration. The GA_3 extract clarified by precipitation was previously tested in germination (data not shown) where it caused the inhibition of seed germination, probably due to the toxicity of the precipitation solution components. Therefore, the purification through micro and ultrafiltration membranes was adopted. After microfiltration and ultrafiltration through 100 kDa and 10 kDa membranes 73% of GA_3 was recovered.

The effect of different treatments (T1, T2, T3, T4, T5 and T6) on soybean seeds germination, root length and number of lateral root, was evaluated (Table 5.1). Seeds treated with GA_3 (T3, T4 and T6) showed higher germination percentage then without treatment (T1) and treated with water (T2). T6 was the best treatment reaching 96% of germinated seeds. Otherwise, the treatment with Gibbtec LS (T5) did not show a positive effect, resulting in only 48% of seed germination. This was probably due to the presence of a high diluent concentration in this formulation. On the other hand, GA_3 extract and Gibbtec SP showed no toxic effect on seeds germination.

It is well known that the applications of GA_3 formulations in seed treatment are linked to the germination induction. The germination process occurs in three steps: water uptake, activation of metabolic process, and radicle emergence. GA_3 function starts in water uptake, where it acts as a negative regulator of germination repressor proteins, inducing the hydrolytic enzymatic complex which catalyses the starch conversion into simple carbohydrates and chemical energy is liberated which is used in the activation of embryo (RAJJOU et al., 2012; PEREIRA et al., 2017). Therefore, these results indicated a positive action of GA_3 application on soybean germination.

Gibbtec SP treated seeds showed the lowest germination time (3.08 days). This treatment shortened seed germination process in 1.17 and 1.24 days comparing to T1 and T2 treatments, respectively, which both do not have GA_3, and in 1.23 days comparing to Progibb® 400 (Table 5.1). Similar results were reported in literature, when

an acceleration in *Phaseolus vulgaris* germination was achieved when GA_3 nanoparticles at 0.7 μg g^{-1} were used (PEREIRA et al., 2017). Zhang et al. (1997) reported that exogenous application of GA_3 accelerate in 1 to 2 days the emergence of soybean seeds under low soil temperature conditions. Moreover, in addition to reducing seed germination time, it was reported that the application of GA_3 in soybean seeds also promoted an enhancement of antioxidant compounds as well as antioxidant activity during germination process (LIEN et al., 2016).

Table 5.1 - Soybean germination assay after 7 days.

	Treatment	Germination (%)	Mean germination time (days)	Root length (cm)	Number of lateral roots
T1	Without immersion	70	4.25	4.48 ± 1.29 (ab)	10.87 ± 5.48 (a)
T2	Deionized water	76	4.32	4.79 ±1.86 (a)	8.39 ± 6.59 (bc)
T3	Progibb® 400	81	3.41	4.69 ± 1.69 (ab)	8.02 ± 6.40 (b)
T4	GA_3 extract	80	3.45	5.13 ± 1.70 (a)	11.35 ± 7.83 (a)
T5	Gibbtec LS	48	4.70	4.07 ± 2.20 (b)	7.33 ± 7.24 (b)
T6	Gibbtec SP	96	3.08	4.95 ± 2.09 (a)	10.54 ± 7.24 (ac)

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

Considering root length, GA_3 extract and Gibbtec SP resulted in larger average root length of 5.13 cm and 4.95 cm, respectively (Table 5.1). However, there was no statistical differences between treatments, except for Gibbtec LS, in which the length was slightly smaller, but there is no statistical difference between treatments without immersion and Progibb® 400. Treatments with GA_3 extract and Gibbtec SP increased lateral root formation when compared to T2, T3 and T5. Furthermore, when GA_3 extract and Gibbtec SP were used, there was an increase of 1.41-fold and 1.32-fold of lateral root formation than using Progibb® 400. No statistically significant difference was obtained compared to the seeds without immersion.

The roots are responsible for plant fixation and for nutrients and water uptake from the soil. In agriculture, a high and strong development of roots is desirable in order to increase the absorption of fertilizers, improving plant quality (LYNCH; BROWN, 2012; PEREIRA et al., 2017). Pereira et al. (2017) working with GA_3 treatment on *Phaseolus vulgaris* seeds, obtained primary roots that were 1.45 cm larger than control and a gain of 74.1% in lateral roots was reached. Application of GA_3 in chickpea promoted an increase of 10% in root length (THAKARE; PATIL; MALPATHAK, 2011). However, in rice seedlings indol-3 butyric acid (IBA) showed better results than GA_3 in

adventitious root formation (WAHYUNI et al., 2003). It is also reported that GA_3 action is essential for root elongation, but high GA_3 concentrations has inhibitory effects (TANIMOTO, 2012; HEDDEN; SPONSEL, 2015).

Soybean germination rate was faster and more efficient using Gibbtec SP than the others treatments (Figure 5.1). In the fourth day, more than 90% of seeds had already been germinated in T6, while in treatments without immersion or immersion in water the percentage of germinated seeds were 43% and 57%, respectively. T3 and T4 showed similar results with more than 70% of germinated seeds in the fourth day. T5 was less efficient and promoted less seed germination. According to Zhang et al. (1997) the optimum temperature for soybean germination is around 25 °C. In this condition, soybean seed requires 4 to 5 days to complete germination, and reaches a germination rate of at least 90%.

For crop production stimulation, a main agricultural goal is to obtain rapid and uniform germination and seedling emergence. In the seed industry, to improve the performance of commercial seed lots, a controlled imbibition of the seeds is performed, aiming to have an advance in germination time compared to untreated seeds (RAJJOU et al., 2012). Therefore, Gibbtec SP that showed promising results related to seed germination, could be employed in pre-treatment of seeds for reduction of germination time and improvement of crop production.

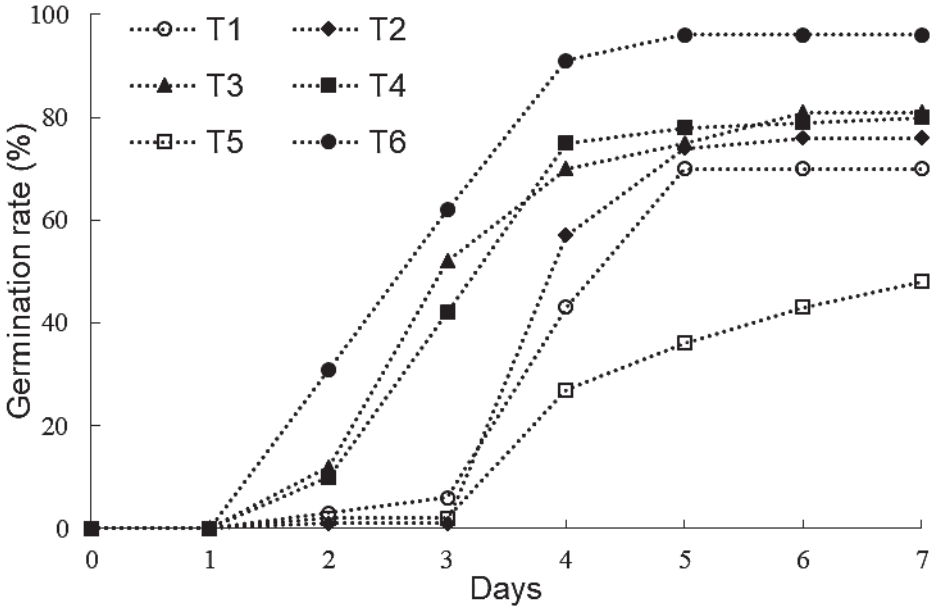


Figure 5.2 - Germination rate of soybean seeds pre-treated during 7 days of incubation period.

GA_3 pre-treatment of soybean seeds also stimulated a faster shoot development when compared with the treatments T1 and T2 (Table 5.2). Progibb® 400 showed shoot length of 4.25 cm, with no statistically significant difference compared to GA_3 extract (3.91 cm) and Gibbtec SP (3.97 cm). Gibbtec LS did not promoted shoot elongation, such as T3, T4 and T6, but the growth was similar, with no significant statistic difference to T1.

Table 5.2 - Shoot development of GA_3 pre-treated soybean seeds.

	Treatment	Shoot development (cm)
T1	Without immersion	2.21 ± 0.64 (c)
T2	Water	1.80 ± 0.54 (b)
T3	Progibb® 400	4.25 ± 1.33 (a)
T4	GA_3 extract	3.91 ± 1.34 (a)
T5	Gibbtec LS	2.21 ± 0.70 (c)
T6	Gibbtec SP	3.97 ± 1.19 (a)

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

In Figure 5.2, it is shown the shoot and root development of soybean seeds after the germination process. The development of all seeds with all treatments is presented in attachment A. Promotion of shoot elongation is one of the most reported effects of GA_3, in which the growth is promoted through cell elongation, due to relaxation of the cell wall (COSGROVE; SOVONICK-DUNFORD, 1989; HEDDEN; SPONSEL, 2015). Similar results were obtained by Wahyuni et al. (2003), Emongor (2007) and Thakare et al. (2011).

Figure 5.3 - Soybean seeds development after 7 days. T1- without immersion; T2- Deionized water; T3- Progibb® 400; T4- GA_3 extract; T5- Gibbtec LS; T6- Gibbtec SP.

As it was seen, Gibbtec SP, which was produced in this work, improved the germination percentage of soybean seeds, shortened the germination time and increased root and lateral root formation when compared to other treatments. The results were better than achieved using the commercial product Progibb® 400. Gibbtec LS was not suitable for seed pre-treatment probably due to high diluent concentration in its formulation, affecting the germination process. GA_3 extract provided promising results, however, low GA_3 stability in aqueous solutions reduce its shelf life and thus, it may be a limiting factor for its application for crop performance improvement. The shelf life of lyophilized Gibbtec SP during 4 months under ambient storage was evaluated, and GA_3 activity remained above 90% during this period. Therefore, Gibbtec SP is a potential product to compete with Progibb® 400 in the seed treatment market.

5.3.2 GA_3 application in soybean plant development

GA_3 preparations were tested in soybean seedlings to evaluate the biological effects of the developed products in plant growth when compared to the Progibb® 400. In the Gibbtec LS formulation was added a surfactant in order to evaluate its effects

during application. The Figure 5.4 shows the effect of surfactant SA, when applied on soybean leaves. In Figure 5.4a, without addition of surfactant, it is possible to notice the presence of droplets on the leaf surface. On the other hand, in Figure 5.4b, it is observed that the formation of droplets does not occur and the applied solution spreads over the entire leaf surface. This occurs due to surfactants act as spreading agent and their main role is to act on the drop surface to reduce the surface tension, improving agrochemicals' performance (CASTRO; OJEDA; CIRELLI, 2014).

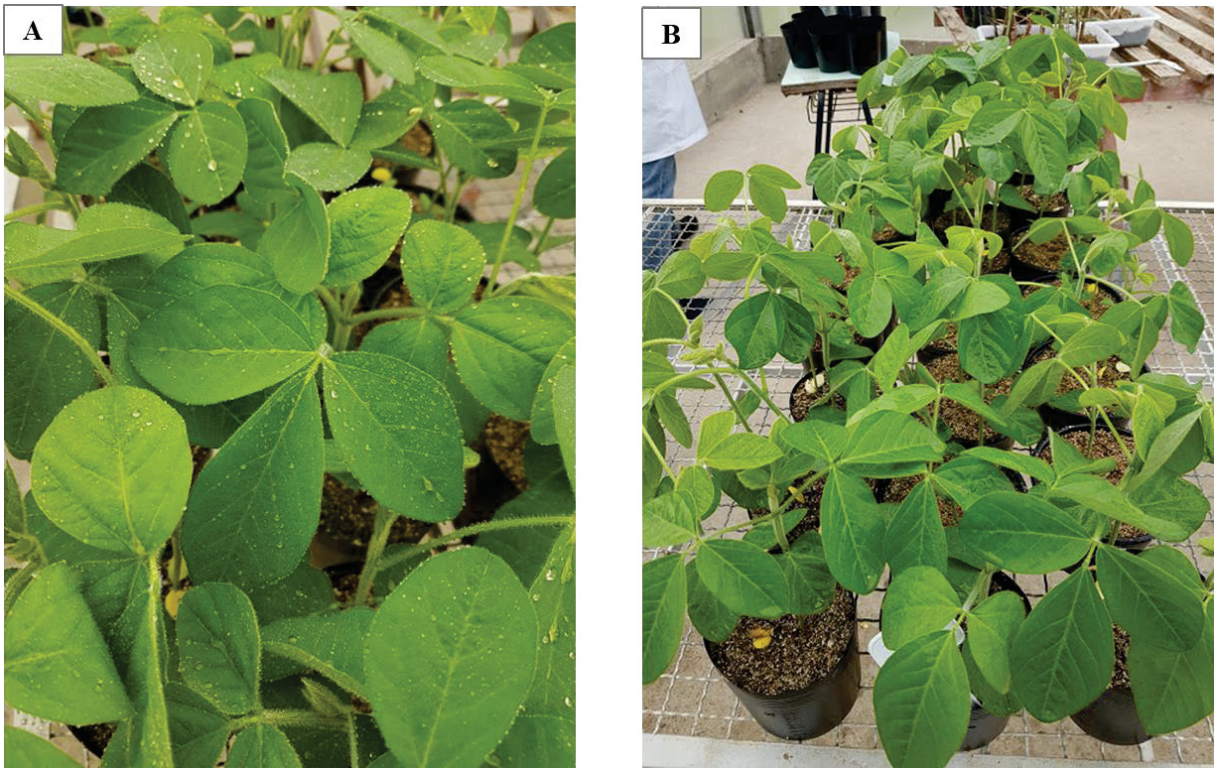


Figure 5.4 – Effect of absence (A) and presence (B) of surfactant SA after application of the products in soybean

GA_3 treatments surely improved soybean development and promoted shoot elongation when compared to the control, the difference can be clearly observed from the fifth day after application (Figure 5.5).

Figure 5.5 - Influence of the GA_3 products application on soybean development 15 days after the first application (V3 stage). T1- Control; T2- Progibb® 400; T3- GA_3 extract; T4- Gibbtec LS; and T5- Gibbtec SP.

The exogenous application of GA_3 products on soybean seedlings significantly promoted stem elongation when compared with control (T1) (Table 5.3). The treatments T2 and T3 showed the highest plant height (143.8 cm and 113.33 cm, respectively), as well as, shoot dry mass, 12.42g in T2 and 11.14g in T3. The developed products, T4 and T5, also promoted stem growth, with no statistical differences between them in plant height and shoot dry mass. However, the treatments T2, T3 and T4 did not improve root development, showing lower root dry mass when compared with T5 and control. Shoot/root ratio also shows that there was a greater development of the aerial part than the roots in the treatments with GA_3 (T2, T3, T4 and T5). T1 presented a lower Shoot/root ratio, suggesting an equilibrium in the development between stem and root

The effect of GA_3 on root growth is indirect, due to its effect on the growth of the aerial part exerted by GA_3 on cell elongation (TANIMOTO, 1990). Therefore, the excessive growth of the aerial part may have influenced the development of the roots. The ideal soybean plants height varies from 60 cm to 110 cm, higher plants could promote lodging, causing field losses at harvest (LEITE; ROSOLEM; RODRIGUES, 2003). Hamayun et al. (2010) and Leite; Rosolem; Rodrigues (2003) observed similar

results when exogenous GA_3 application improved soybean stem height, but did not show significant effects in root dry mass.

Table 5.3 - Effects of different GA_3 treatments in soybean development.

Treatments		Plant high (cm)	Shoot dry mass (g)	Root dry mass (g)	Shoot/root ratio (g)
T1	Water	61.60 ± 4.20 (d)	9.86 ± 1.89 (a)	9.96 ± 5.51 (b)	1.42 ± 1.03 (a)
T2	Progibb	143.80 ± 25.13 (a)	12.42 ± 3.78 (b)	4.85 ± 2.58 (a)	3.15±1.42 (bc)
T3	Extract	113.33 ± 17.28 (b)	11.14 ± 1.59 (ab)	4.80 ± 2.61 (a)	2.96 ± 1.47 (bc)
T4	Gibbtec LS	102.37 ± 16.5 (c)	9.72 ± 1.41 (a)	3.88 ± 2.29 (a)	3.37 ± 1.87 (b)
T5	Gibbtec SP	105.03 ± 18.25 (c)	10.23 ± 2.58 (a)	9.75 ± 10.22 (b)	2.18 ± 1.79 (ac)

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

GA_3 treatments were not effective in increasing soybean productivity when compared to control (T1) (Table 5.4). T1 showed the highest number of pods (685) and grains (1364). However, among treatments containing GA_3, the GA_3 extract and Gibbtec SP showed higher total number of seeds than Progibb® 400 (986, 914 and 901, respectively), and no statistical difference among them in relation with pods and grains per plant. Although the control had a greater grain production, the 1000-grain weight presented similar results when compared to the weight in treatments with GA_3, indicating that the application of GA_3 influenced in the filling of the grains instead of grain production, since smaller amounts of grains and heavier ones were obtained. This result was more pronounced in treatments T2, T3 and T4.

Table 5.4 - Effects of GA_3 treatments in soybean productivity.

Treatments		Pods		Grains			1.000-grain weight (g)
		Pods/plant	*Total*	*Grains/pods*	*Grains/plant*	*Total*	
T1	Water	17 ± 6.74 (a)	685	2.0 ± 0.24 (ab)	34.10 ± 13.33 (a)	1364	92,65
T2	Progibb® 400	12 ± 5.14 (bc)	477	1.85 ± 0.27 (b)	22.5 ± 10.81 (bc)	901	88,05
T3	Extract	12.5 ± 8.65 (b)	486	2.08 ± 0.35 (a)	25 ± 8.65 (b)	986	90,03
T4	Gibbtec LS	10 ± 4.75 (c)	402	1.97 ± 0.25 (ab)	19.65 ± 9.22 (c)	786	87,67
T5	Gibbtec SP	12 ± 4.06 (bc)	473	1.95 ± 0.36 (ab)	23 ± 7.91 (bc)	914	74,29

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

Regarding seed per pod, all treatments showed similar results (Table 5.4). According to Lee and Herbek (2005), healthy soybean plants should present about 2.5

seeds per pod. For soybeans under stress, the seeds per pod could drop to 2.0, 1.5, or even less under high stress situations. It was observed, during the assay, a disease caused by the fungi *Microsphaera diffusa* on soybean plants. The disease can cause a stress on plant, resulting in early defoliation of soybean leaves that result in reduced weight of the seeds and pods ranging from 10% to 90% depending on the phase of plant development, environment condition and soybean varieties (YULIA et al., 2017). Thus, this disease probably caused a stress in these soybean plants, which can also affected the grains production, and therefore it was not possible to conclude the effect of GA_3 containing products on soybean productivity.

There are some reports on literature showing that GA_3 application in soybean did not interfere in increasing soybean production. Mislevy; Boote; Martin (1989) reported that foliar GA_3 application in the “craking” stage of soybean did not effect on seed yield. Moreover, Zhang; Pan; Smith (1997) showed that GA_3 application in soybean seeds at the time of planting did not influence final grain yield also. These results suggested that GA_3 application in vegetative stages of soybean did not affect on soybean productivity, acting only in stem elongation. Hence, further studies are needed to evaluate to determine optimum concentrations and timing of application to improve soybean yield.

Nevertheless, the developed products, Gibbtec LS and Gibbtec SP, showed similar biological activity in promoting soybean stem elongation when compared to the commercial product Progibb® 400. They stimulated vegetative development of the plants and clearly can be used in crops in which stem elongation is necessary. Moreover, the presence of diluent in Gibbtec LS formulation did not affect the plants and the presence of surfactant could improve GA_3 absorption in plant leaves. The developed preparations were obtained through a cheap bioprocess, reducing its production cost and becoming a competitive product for national and international market.

5.4 CONCLUSIONS

The current study tested a liquid (Gibbtec LS) and powder (Gibbtec SP) GA_3 formulations. GA_3 was obtained through semi-solid state fermentation using a simple fermentation medium that resulted in a reduction on its production costs. Gibbtec LS

showed some toxic effects on seed germination probably due to the high diluent concentration in its formulation, but any toxic effect was observed when applied in foliar treatment. Gibbtec SP showed promising results for all evaluated parameters in the germination of soybean seeds and for plant growth stimulation. Moreover, Gibbtec SP presented better or similar results to that found using the available commercial product, Progibb® 400. These results demonstrate that Gibbtec SP and Gibbtec LS have potential to be used in agricultural industry for crop stimulation.

CONCLUSIONS

This research worked with an important plant growth hormone, responsible for different responses on vegetal development. The GA_3 was produced by *Fusarium fujikuroi* LPB 06 using Brazillian agroindustrial subproducs. The use of a mixture of citrus pulp (CP) and soybean hulls (SH) to compose the culture medium result in a GA_3 production of 415 mg L^{-1} (3.75 mg L^{-1} h^{-1}) in Erlenmeyer flasks, 279.66 mg L^{-1} in bubble column reactor (BCR) (1.66 mg $L^{-1}h^{-1}$), and 205 mg L^{-1} in stirred tank reactor (STR) (2.14 mg $L^{-1}h^{-1}$). It is also showed that the GA_3 production using complex culture medium was 2.55-fold and 2.20-fold higher than using sintetic medium in BCR and STR, respectively. This results shows that CP and SH can be used in culture medium as alternative carbon and nitrogen source for GA_3 production, lowing the production costs. The process scale-up in BCR and STR still need more studies concerning to fermentation paramenters in order to improve GA_3 production.

GA_3 extract obtained after fermentation was the focus of studies to compose stable formulations. The extract clarification process through precipitation showed toxic effects when applied in seeds. Therefore, a clarification method using microfiltration (MF) and ultrafiltration (UF) membranes was adoped, achieving a GA_3 recovery of 73% after MF and UF in 100 kDa and 10 KDa. GA_3 extract stability was evaluated under accelerated conditions (54 °C for 14 days) and ambient conditions (6 months), with 67% and 45% of GA_3 activity was maintened, respectively.

Thus, the results confirms that GA_3 has low stability in aqueous solution, being necessary a formulation step to ensure higher shelf life for the product. In liquid formulation tests, using 50% of DX and 2.5% of SA showed the best effects in maintained GA_3 stability. This condition maintained 67% of GA_3 activity at 54 °C for 14 days and 99% after 6 months under ambient storage condition. Powder formulation studies showed that the best condition was obtained using 10% of CA and drying at 180 °C in spray dryer. GA_3 stability was mainteined during 8 months. Extract lyophilization with 10% of CA mainteined the GA_3 stability for 4 months.

The developed products were applied in soybean seeds and in soybean seedlings in three different stages of development. It was found that GA_3 products application stimulated seed and plant development. Seeds pre-treatment with GA_3 lowering the mean germination time increased the germination porcetage and improve

shoot elongation when compared to the treatments without GA_3. Gibbtec SP showed the best results, achieving 96% of germinated seeds in 5 days, with mean germination time of 3.08 days. GA_3 extract and Progibb® 400, showed similar results regarding germination percentage (80% and 81%) and mean germination time (3.45 and 3.41 days). No statiscally significant diference were observed in root length and lateral roots between treatments. Gibbtec LS showed inhibitory effects for seed germination due to high diluent concentration. The studies involving plant development showed that foliar GA_3 applications significantly increase stem elongation and shoot dry mass. However, did not affect root dry mass and soybean grains production. More studies are necessary aiming to improve soybean yields.

Therefore, this work showed that it is possible to use the agroindustrial subproducts citrus pulp and soybean hulls for GA_3 production, resulting in an economic and sustainable production. Moreover, through the screening of different additives and diluents, it was possible to obtain stable liquid and powder formulations, in which the GA_3 effects related to plant development, could be seen in soybean plants. Nevertheless, the developed products have potencial to be used in agroindustry, in different cultivars, for crop improvement.

FUTURE PROSPECTS

Researches about GA_3 have been conducted since the 1930s. However, the main bottleneck is still its low production, which consequently increases its cost. Thus, researchers are constantly searching for new forms of production. The fermentation technique used in this work is still little studied and therefore, scale-up studies could be an interesting strategy to improve GA_3 production by *Fusarium fujikuroi*. Downstream studies, aiming to reduce losses and costs, as well as increase the concentration of the molecule of interest, are necessary. Moreover, the lack of information about stability and formulation studies opens the opportunity for searching new ingredients that are capable of maintaining the stability of the liquid and powder products, reducing toxic effects and improving GA_3 activity on plant development. New studies to evaluate the effects of exogenous application of GA_3 developed products on different economic important crops, under field conditions and even testing the tolerance against abiotic stress could elucidate some aspects of plant production.

REFERENCES

AGOSIN, E. et al. Production of gibberellins by solid substrate cultivation of *Gibberella fujikuroi*. **Advances in Solid State Fermentation**, p. 355–366, 1997.

AKTER, N. et al. Alleviation of drought stress in maize by exogenous application of gibberellic acid and cytokinin. **Journal of Crop Science and Biotechnology**, v. 17, n. 1, p. 41–48, 2014.

ALBERMANN, S. et al. Characterization of novel mutants with an altered gibberellin spectrum in comparison to different wild-type strains of *Fusarium fujikuroi*. **Applied Microbiology and Biotechnology**, v. 97, n. 17, p. 7779–7790, 2013.

ALVARENGA, R. et al. Induction of resistance of corn plants to *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae) by application of silicon and gibberellic acid. **Bulletin of Entomological Research**, p. 1–7, 2017.

ANANDHARAMAKRISHNAN, C.; RIELLY, C. D.; STAPLEY, A. G. F. Loss of solubility of α-lactalbumin and β-lactoglobulin during the spray drying of whey proteins. **LWT - Food Science and Technology**, v. 41, n. 2, p. 270–277, 2008.

ATAY, A. N.; KOYUNCU, F. Manipulating Regular Bearing in 'Golden Delicious'/M9 Apple Trees using GA4+7 and Ethephon. **International Journal of Fruit Science**, v. 16, n. 1, p. 10–22, 2016.

ATES, S.; OZENIR, S.; GÖKDERE, M. Effect of silicone oil on gibberellic acid production by *Gibberella fujikuroi* and *Aspergillus niger*. **Applied Biochemistry and Microbiology**, v. 42, n. 5, p. 500–501, set. 2006.

BALAGUERA-LOPEZ, H. E.; CARDENAS-HERNANDEZ, J. F.; ALVAREZ-HERRERA, J. G. Effect of gibberellic acid (GA3) on seed germination and growth of tomato (*Solanum lycopersicum* L.). **Acta Hort.(ISHS)**, v. 821, p. 141–148, 2009.

BALASUBRAMANI, P. et al. Microencapsulation of garlic oleoresin using maltodextrin as wall material by spray drying technology. **International Journal of Biological Macromolecules**, v. 72, p. 210–217, 2015.

BANDELIER, S.; RENAUD, R.; DURAND, A. Production of gibberellic acid by fed-batch solid state fermentation in an aseptic pilot-scale reactor. **Process Biochemistry**, v. 32, n. 2, p. 141–145, 1997.

BELGHITH, H.; ELLOUZ CHAABOUNI, S.; GARGOURI, A. Stabilization of *Penicillium occitanis* cellulases by spray drying in presence of maltodextrin. **Enzyme and**

Microbial Technology, v. 28, n. 2–3, p. 253–258, 2001.

BERRÍOS, J.; ILLANES, A.; AROCA, G. Spectrophotometric method for determining gibberellic acid in fermentation broths. **Biotechnology Letters**, v. 26, p. 67–70, 2004.

BERRIOS, J.; PYLE, D. L.; AROCA, G. Gibberellic acid extraction from aqueous solutions and fermentation broths by using emulsion liquid membranes. **Journal of Membrane Science**, v. 348, n. 1–2, p. 91–98, fev. 2010.

BÖMKE, C.; TUDZYNSKI, B. Diversity, regulation, and evolution of the gibberellin biosynthetic pathway in fungi compared to plants and bacteria. **Phytochemistry**, v. 70, p. 1876–1893, 2009.

BORROW, A. et al. The effect of varied temperature on the kinetics of metabolism of *Gibberella fujikuroi* in stirred culture. **Canadian Journal of Microbiology**, v. 10, n. 3, p. 445–466, 1964.

BRIAN, P. W. et al. **Gibberellic acid compounds, and preparation and use thereof**, US2842051, 1958.

BRIGGS, D. E. Brewing industry research foundation effects of gibberellic acid on barley germination and its use in malting: a review. **Journal of The institute of brewing**, v. 69, p. 244–248, 1963.

BRUCKNER, B. et al. The Gibberellin Fermentation. **Critical Reviews in Biotechnology**, v. 192, p. 163–192, 1991.

CANDAU, R.; AVALOS, J.; CERDÁ-OLMEDO, E. Regulation of gibberellin biosynthesis in *Gibberella fujikuroi*. **Plant Physiology**, v. 100, n. 3, p. 1184–1188, 1992.

CANO-CHAUCA, M. et al. Effect of the carriers on the microstructure of mango powder obtained by spray drying and its functional characterization. **Innovative Food Science and Emerging Technologies**, v. 6, n. 4, p. 420–428, 2005.

CARNEIRO, H. C. F. et al. Encapsulation efficiency and oxidative stability of flaxseed oil microencapsulated by spray drying using different combinations of wall materials. **Journal of Food Engineering**, v. 115, n. 4, p. 443–451, 2013.

CARVALHO, C. DE et al. Brazilian Fruit Yearbook. **Editora Gazeta**, p. 88, 2017.

CASANOVA, L. et al. The application of gibberellic acid increases berry size of 'Emperatriz' seedless grape. **Spanish Journal of Agricultural Research**, v. 7, n. 4, p. 919–927, 2009a.

CASANOVA, L. et al. Scoring increases carbohydrate availability and berry size in

seedless grape “Emperatriz”. **Scientia Horticulturae**, v. 122, p. 62–68, 2009b.

CASTRO, M. J. L.; OJEDA, C.; CIRELLI, A. F. Advances in surfactants for agrochemicals. **Environmental Chemistry Letters**, v. 12, n. 1, p. 85–95, 2014.

CHAVEZ-PARGA, M. C. et al. Hydrodynamics, mass transfer and rheological studies of gibberellic acid production in an airlift bioreactor. **World Journal of Microbiology and Biotechnology**, v. 23, n. 5, p. 615–623, 23 set. 2006.

CHAVEZ-PARGA, M. D. C. et al. Kinetic of the gibberellic acid and bikaverin production in an airlift bioreactor. **Process Biochemistry**, v. 43, n. 8, p. 855–860, ago. 2008.

CHEN, S.-Y.; KUO, S.-R.; CHIEN, C.-T. Roles of gibberellins and abscisic acid in dormancy and germination of red bayberry (*Myrica rubra*) seeds. **Tree Physiology**, v. 28, n. 9, p. 1431–1439, 1 jul. 2008.

CHIOU, D.; LANGRISH, T. A. G. Crystallization of amorphous components in spray-dried powders. **Drying Technology**, v. 25, n. 9, p. 1423–1431, 2007.

CHRISTIAENS, A. et al. Flower development and effects of a cold treatment and a supplemental gibberellic acid application on flowering of *Helleborus niger* and *Helleborus x ericsmithii*. **Scientia Horticulturae**, v. 136, p. 145–151, 2012.

COLEBROOK, E. H. et al. The role of gibberellin signalling in plant responses to abiotic stress. **The Journal of experimental biology**, v. 217, n. Pt 1, p. 67–75, 1 jan. 2014.

CONAB. Acompanhamento da safra brasileira de grãos. **v. 9 Safra 2015/16**, p. 1–178, 2016.

CONAB. **Acompanhamento da safra brasileira de grãos**. Disponível em: <http://www.conab.gov.br>.

COSGROVE, D. J.; SOVONICK-DUNFORD, S. A. Mechanism of gibberellin-dependent stem elongation in peas. **Plant physiology**, v. 89, p. 184–91, 1989.

DATTA, R.; VASEK, G. J.; PASIETA, L. M. **Liquid formulation of a plant growth regulator**, US 0172890, 2006.

DAYAN, J. et al. Leaf-induced gibberellin signaling is essential for internode elongation, cambial activity, and fiber differentiation in Tobacco stems. **The Plant Cell**, v. 24, n. 1, p. 66–79, 2012.

DEVISETTY, B. N. et al. **Concentrated, Water-soluble, Granular plant growth regulator formulation and methods for use of same**, US0172890, 2006.

DEVISETTY, B. N. et al. Development of fast dissolving concentrated gibberellin. **Journal of ASTM International**, v. 4, n. 3, p. 3–10, 2007.

DEVISETTY, B. N. et al. **Low voc gibberellins formulations.** US 20150173365, 2015.

ECONOMOU, C. N. et al. Semi-solid state fermentation of sweet sorghum for the biotechnological production of single cell oil. **Bioresource Technology**, v. 101, n. 4, p. 1385–1388, 2010.

EMONGOR, V. Gibberellic acid (GA3) influence on vegetative growth, nodulation and yield of cowpea (*Vigna unguiculata* (L.). **Walp.Journal of Agronomy**, 2007.

ESCAMILLA, E. M. et al. Optimization of gibberellic acid production by immobilized *Gibberella fujikuroi* mycelium in fluidized bioreactors. **Journal of biotechnology**, v. 76, p. 147–155, 2000.

ESCAMILLA SILVA, E. M. et al. Morphological development and gibberellin production by different strains of *Gibberella fujikuroi* in shake flasks and bioreactor. **World Journal of Microbiology and Biotechnology**, v. 15, p. 753–755, 1999.

FAO. **Manual on development and use of FAO and WHO specifications for pesticides**, 2010.

FINCH-SAVAGE WE, LEUBNER-METZGER G. Seed dormancy and the control of germination. **New Phytol** 171:501–23, 2006.

GEARY, R. J.; BEACH, V.; HABER, R. **Plant Growth Regulator**, US3038794, 1962.

GONZALEZ, J. C. et al. Production of *Trametes pubescens* laccase under submerged and semi-solid culture conditions on agro-industrial wastes. **PLoS ONE**, v. 8, n. 9, p. 1–14, 2013.

HAMAYUN, M. et al. Exogenous gibberellic acid reprograms soybean to higher growth and salt stress tolerance. **Journal of Agricultural and Food Chemistry**, v. 58, p. 7226–7230, 2010.

HAO, Y. H. et al. Stable isotope labeling assisted liquid chromatography-electrospray tandem mass spectrometry for quantitative analysis of endogenous gibberellins. **Talanta**, v. 144, p. 341–348, 2015.

HEDDEN, P.; PHILLIPS, A. L. Gibberellin metabolism: new insights revealed by the genes. **Trends in Plant Science**, v. 5, n. 12, p. 523–530, dez. 2000.

HEDDEN, P.; SPONSEL, V. A Century of Gibberellin Research. **Journal of Plant Growth Regulation**, v. 34, n. 4, p. 740–760, 2015.

HOAGLAND, D. R.; ARNON, D. I. The water-culture method for growing plants without soil. **California Agricultural Experiment Station Circular**, v. 347, n. 347, p. 1–32, 1950.

HOLBROOK, A.; EDGE, W.; BAILEY, F. Spectrophotometric method for determination of gibberellic acid. **Adv Chem Ser**, v. 28, p. 159–167, 1961.

HOLLMANN, D. et al. Extractive fermentation of gibberellic acid by *Gibberella fujikuroi*. **Journal of Fermentation and Bioengineering**, v. 79, n. 6, p. 594–600, 1995a.

HOLLMANN, D. et al. Extractive fermentation of gibberellic acid by *Gibberella fujikuroi*. **Journal of Fermentation and Bioengineering**, v. 79, n. 6, p. 594–600, 1995b.

HORVITZ, S. et al. Application of gibberellic acid to'Sweetheart'sweet cherries: effects on fruit quality at harvest and during cold storage. **XXVI International Horticultural Congress: Issues and Advances in Postharvest Horticulture**, v. 628, p. 311–316, 2002.

JAFARI, S. M. et al. Encapsulation efficiency of food flavours and oils during spray drying. **Drying Technology**, v. 26, n. 7, p. 816–835, 2008.

KAHLON, S. S.; MALHOTRA, S. Production of gibberellic acid by fungal mycelium immobilized in sodium alginate. **Enzyme and Microbial Technology**, v. 8, n. 10, p. 613–616, 1986.

KARR-LILIENTHAL, L. K. et al. Chemical and nutritional properties of soybean carbohydrates as related to nonruminants : A review. **Livestock Production Science**, v. 97, p. 1–12, 2005.

KARTHIK, P.; ANANDHARAMAKRISHNAN, C. Microencapsulation of Docosahexaenoic Acid by Spray-Freeze-Drying Method and Comparison of its Stability with Spray-Drying and Freeze-Drying Methods. **Food and Bioprocess Technology**, v. 6, n. 10, p. 2780–2790, 2013.

KAVANAGH, F.; KUZEL, N. R. Fluorometric determination of gibberellic acid and gibberellenic acids in fermentation products, commercial formulations, and purified materials. **Agricultural and Food Chemistry**, v. 6, p. 459–463, 1958.

KAWAIDE, H. K. Biochemical and molecular analyses of gibberellin biosynthesis in fungi. **Bioscience Biotechnology Biochemistry**, v. 70, n. 3, p. 583–590, 2006.

KOYUNCU, F. Breaking seed dormancy in black mulberry (*Morus nigra* L.) by cold stratification and exogenous application of gibberellic acid. **Acta Biologica Cracoviensia Series Botanica**, v. 47, n. 2, p. 23–26, 2005.

KUMAR, P. K. R.; LONSANE, B. K. Solid state fermentation : physical and nutritional factors influencing gibberellic acid production. **Applied Microbiology and Biotechnology**, v. 34, p. 145–148, 1990.

KUMAR, P. K. R.; SANKAR, K. U.; LONSANE, B. K. Supercritical fluid extraction from dry mouldy bran for the purification of gibberellic acid from the concomitant products produced during solid state fermentation. **The Chemical Engineering Journal**, v. 46, n. 2, p. 53–58, 1991.

LA PIERRE, R. J.; ISELIN, N. J. **Stable Formulations of Plant Growth Stimulant**, US3004845, 1961.

LALE, G.; GADRE, R. Enhanced production of gibberellin A4 (GA4) by a mutant of *Gibberella fujikuroi* in wheat gluten medium. **Journal of Industrial Microbiology and Biotechnology**, v. 37, n. 3, p. 297–306, 2010.

LEE, C.; HERBEK, J. Estimating Soybean Yield. **University of Kentucky.**, v. agr-188, n. 12–2005, p. 7–8, 2005.

LEITE, V. M.; ROSOLEM, C. A.; RODRIGUES, J. D. Gibberellin and cytokinin effects on soybean growth. **Scientia Agricola**, v. 60, n. 3, p. 537–541, 2003.

LI, Y.-H. et al. Exogenous gibberellic acid increases the fruit weight of 'Comte de Paris' pineapple by enlarging flesh cells without negative effects on fruit quality. **Acta Physiologiae Plantarum**, v. 33, n. 5, p. 1715–1722, 18 jan. 2011.

LIEN, D. T. P. et al. Time and temperature dependence of antioxidant activity from soybean seeds (*Glycine max* L. Merr.) during germination. **International Journal of Food Science and Nutrition**, v. 1, n. 5, p. 22–27, 2016.

LIU, Y. et al. Synthesis and characterization of gibberellin-chitosan conjugate for controlled-release applications. **International journal of biological macromolecules**, v. 57, p. 213–7, jun. 2013.

LOKSUWAN, J. Characteristics of microencapsulated β-carotene formed by spray drying with modified tapioca starch, native tapioca starch and maltodextrin. **Food Hydrocolloids**, v. 21, n. 5–6, p. 928–935, 2007.

LU, X. Z.; XIE, Z. C.; KUMAKURA, M. Production of Gibberellic Acid in *Gibberella fujikuroi* Adhered onto Polymeric Fibrous Carriers. **Process Biochemistry**, v. 30, n. 7, p. 661–665, 1995.

LU, Y. H. et al. Determination of gibberellins using HPLC coupled with fluorescence detection. **Analytical Methods**, v. 8, n. 7, p. 1520–1526, 2016.

LYNCH, J. P.; BROWN, K. M. New roots for agriculture: exploiting the root phenome. **Philosophical Transactions of the Royal Society B: Biological Sciences**, v. 367, n. 1595, p. 1598–1604, 2012.

MACHADO, C. M. M. et al. Coffee husk as substrate for the production of gibberellic acid by fermentation. In: **Book on Coffee Biotechnology and Quality, Sera T., Soccol C. R, Pandey A. and Roussos S. (eds)**. Dorcrecht: Kluwer Academic Publishers, 2001. p. 401–8.

MACHADO, C. M. M. et al. Gibberellic acid production by solid-state fermentation in coffee husk. **Applied Biochemistry and Biotechnology**, v. 102–103, p. 179–191, 2002.

MACLEOD, A. M.; MILLAR, A. S. Effects of Gibberellic Acid on Barley Endosperm. **Journal of the Institute of Brewing**, v. 68, n. 4, p. 322–332, 1962.

MACMILLAN, J. Occurrence of gibberellins in vascular plants, fungi, and bacteria. **Journal of Plant Growth Regulation**, v. 20, p. 387–442, 2001.

MAGALHÃES, A. I. et al. Downstream process development in biotechnological itaconic acid manufacturing. **Applied Microbiology and Biotechnology**, v. 101, n. 1, p. 1–12, 2017.

MAI, H. T. N.; LEE, K. M.; CHOI, S. S. Enhanced oxalic acid production from corncob by a methanol-resistant strain of *Aspergillus niger* using semi solid-sate fermentation. **Process Biochemistry**, v. 51, n. 1, p. 9–15, 2016.

MARTIN, A. .; DOBRAT, W. CIPAC Handbook Volume F. In: **Collaborative International Pesticides Analytical Council Ltd.** Harpenden, U.K.: [s.n.].

MEDINA-TORRES, L. et al. Microencapsulation by spray drying of gallic acid with nopal mucilage (*Opuntia ficus indica*). **LWT - Food Science and Technology**, v. 50, n. 2, p. 642–650, 2013.

MELEIGY, S. A.; KHALAF, M. A. Biosynthesis of gibberellic acid from milk permeate in repeated batch operation by a mutant *Fusarium moniliforme* cells immobilized on loofa sponge. **Bioresource Technology**, v. 100, p. 374–379, 2009.

MIELENZ, J. R.; BARDSLEY, J. S.; WYMAN, C. E. Fermentation of soybean hulls to ethanol while preserving protein value. **Bioresource Technology**, v. 100, n. 14, p. 3532–3539, 2009.

MISLEVY, P.; BOOTE, K. J.; MARTIN, F. G. Soybean response to gibberellic acid treatments. **Journal of Plant Growth Regulation**, v. 8, p. 11–18, 1989.

MONTIBELLER, V. W. et al. Characterization of Hemicellulolytic Enzymes Produced by *Aspergillus niger* NRRL 328 under Solid State Fermentation on Soybean Husks. **Bioresources**, v. 9, n. 4, p. 7128–7140, 2014.

MUÑOZ-FAMBUENA, N. et al. Gibberellic Acid Reduces Flowering Intensity in Sweet Orange [*Citrus sinensis* (L.) Osbeck] by Repressing CiFT Gene Expression. **Journal of Plant Growth Regulation**, v. 31, n. 4, p. 529–536, 2012.

NASRI, N. et al. Effect of exogenous gibberellic acid on germination, seedling growth and phosphatase activities in Lettuce under salt stress. **African Journal of Biotechnology**, v. 11, n. 56, p. 11967–11971, 2012.

NELSON, N. A photometric adaptation of the Somogyi method for the determination of glucose. **Journal of Biological Chemistry**, v. 153, p. 375–380, 1944.

NHUJAK, T. et al. Determination of gibberellic acid in fermentation broth and commercial products by micellar electrokinetic chromatography. **J Agric Food Chem**, v. 53, n. 6, p. 1884–1889, 2005.

OLIVEIRA, J. DE et al. Gibberellic acid production by different fermentation systems using citric pulp as substrate / support. **Biomed Research International**, v. 2017, p. 8, 2017.

OLIVEIRA, J. **Produção de ácido giberélico por fermentação submersa e por fermentação no estado semissólido em diferentes modelos de biorreatores utilizando polpa cítrica**. Federal University of Paraná, 2012.

ORTEGA-BAES, P.; ROJAS-ARÉCHIGA, M. Seed germination of *Trichocereus terscheckii* (Cactaceae): Light, temperature and gibberellic acid effects. **Journal of Arid Environments**, v. 69, n. 1, p. 169–176, 2007.

PANDEY, A.; SOCCOL, C. R.; MITCHELL, D. New developments in solid state fermentation: I-bioprocesses and products. **Process Biochemistry**, v. 35, n. 10, p. 1153–1169, jul. 2000.

PASTRANA, L. M. et al. A fed-batch culture model for improved production of gibberellic acid from a waste medium. **Biotechnology Letters**, v. 17, n. 3, p. 263–268, mar. 1995.

PAWLAK, J. A. **Gibberellin formulations**, US 20160360748, 2016.

PEREIRA, A. E. S. et al. γ-Polyglutamic acid/chitosan nanoparticles for the plant growth regulator gibberellic acid: Characterization and evaluation of biological activity. **Carbohydrate Polymers**, v. 157, p. 1862–1873, 2017.

PÉREZ-JIMÉNEZ, M. et al. Regulation of the drought response of sweet pepper (Capsicum annuum L.) by foliar-applied hormones, in Mediterranean-climate greenhouse conditions. **Plant Growth Regulation**, v. 80, n. 2, p. 159–169, 2016.

PÉREZ, F. J. et al. Gibberellic acid decomposition and its loss of biological activity in aqueous solutions. **Phytochemistry**, v. 41, n. 3, p. 675–679, 1996.

RADEMACHER, W. Gibberellin formation in microorganisms. **Plant Growth Regulation**, v. 15, n. 3, p. 303–314, 1994.

RADEMACHER, W. et al. **Plant growth regulating formulations**, WO/2000/002454A1, 1999.

RADEMACHER, W. Growth Retardants: Effects on Gibberellin Biosynthesis and Other Metabolic Pathways. **Plant Physiology and Plant Molecular Biology**, v. 51, p. 501–531, 2000.

RADEMACHER, W. Plant Growth Regulators: Backgrounds and Uses in Plant Production. **Journal of Plant Growth Regulation**, v. 34, n. 4, p. 845–872, 2015.

RADY, M. M.; MOHAMED, G. F. Modulation of salt stress effects on the growth, physio-chemical attributes and yields of *Phaseolus vulgaris* L. plants by the combined application of salicylic acid and *Moringa oleifera* leaf extract. **Scientia Horticulturae**, v. 193, p. 105–113, 2015.

RAJABI, H. et al. Retention of saffron bioactive components by spray drying encapsulation using maltodextrin, gum arabic and gelatin as wall materials. **Food Hydrocolloids**, v. 51, p. 327–337, 2015.

RAJJOU, L. et al. Seed Germination and Vigor. **Annual Review of Plant Biology**, v. 63, n. 1, p. 507–533, 2012.

RANGASWAMY, V. Improved Production of Gibberellic Acid by *Fusarium moniliforme* . **Journal of Microbiology Research**, v. 2, n. 3, p. 51–55, 9 maio 2012.

RIOS-IRIBE, E. Y. et al. Inductive effect produced by a mixture of carbon source in the production of gibberellic acid by *Gibberella fujikuroi*. **World journal of microbiology & biotechnology**, v. 27, n. 6, p. 1499–505, jun. 2011.

RIOS-IRIBE, E. Y.; HERNÁNDEZ-CALDERÓN, O. M.; ESCAMILLA-SILVA, E. M. Kinetic analysis of the uptake of glucose and corn oil used as carbon sources in batch cultures of *Gibberella fujikuroi*. **World journal of microbiology & biotechnology**, v. 32, p. 182, 2016.

RODRIGUES, C. et al. A new alternative to produce gibberellic acid by solid state fermentation. **Brazilian Archives of Biology and Technology**, v. 52, n. November, p. 181–188, 2009a.

RODRIGUES, C. et al. Improvement on citric acid production in solid-state

fermentation by *Aspergillus niger* LPB BC mutant using citric pulp. **Applied biochemistry and biotechnology**, v. 158, n. 1, p. 72–87, jul. 2009b.

RODRIGUES, C. **Produção, extração e purificação de hormônio vegetal (ácido giberélico) por fermentação no estado sólido em polpa cítrica e utilização do extrato fermentado em meio de cultivo de bromélias in vitro**. Universidade Federal do Paraná, 2010.

RODRIGUES, C. et al. **Processo de produção e purificação de ácido giberélico, seu uso e aplicações.**, 2012b.

RODRIGUES, C. et al. New perspectives of gibberellic acid production: a review. **Critical reviews in biotechnology**, v. 32, n. 3, p. 263–73, set. 2012a.

RODRIGUES, C. et al. Production , extraction and Purification of Gibberellic Acid by Solid State Fermentation Using Citric Pulp and Soy Husk. **Baoj Chemistry**, v. 2, n. 2, p. 8, 2016.

SALEEM, M. et al. Gibberellic acid in combination with pressmud enhances the growth of sunflower and stabilizes chromium (VI) -contaminated soil. **Environmental Sci Pollut Res**, v. 22, p. 10610–10617, 2015.

SATPUTE, D.; SHARMA, V.; MURARKAR, K. Solid-state fermentation for production of gibberellic acid using agricultural residues. **International Journal Enviroment and Pollution**, v. 43, p. 201–213, 2010.

SELVARAJ, S.; MURTY, V. R. Semi-solid state fermentation: A promising method for production and optimization of tannase from *Bacillus gottheilii* M2S2. **Research Journal of Biotechnology**, v. 12, n. 4, p. 39–48, 2017.

SENIOR, N.; PARK, A. **Dry Formulations of Gibberellic acid Compounds**, US 3031290, 1962.

SHARMA, R. et al. Determination of gibberellins in fermentation broth produced by *Fusarium verticilliodes* MTCC 156 by high-performance liquid chromatography tandem mass spectrometry. **Biotechnology Applied Biochemistry**, v. 88, p. 83–88, 2004.

SHARMA, R. R.; SINGH, R. Gibberellic acid influences the production of malformed and button berries, and fruit yield and quality in strawberry (Fragaria×ananassa Duch.). **Scientia Horticulturae**, v. 119, n. 4, p. 430–433, fev. 2009.

SHOMEILI, M. et al. Effects of gibberellic acid on sugarcane plants exposed to salinity under a hydroponic system. v. 5, n. 10, p. 609–616, 2011.

SHUKLA, R.; CHAND, S.; SRIVASTAVA, A. K. Batch kinetics and modeling of

gibberellic acid production by *Gibberella fujikuroi*. **Enzyme and Microbial Technology**, v. 36, n. 4, p. 492–497, mar. 2005a.

SHUKLA, R.; CHAND, S.; SRIVASTAVA, A. K. Improvement of gibberellic acid production using a model based fed-batch cultivation of *Gibberella fujikuroi.* **Process Biochemistry**, v. 40, n. 6, p. 2045–2050, maio 2005b.

SHUKLA, R.; CHAND, S.; SRIVASTAVA, A. K. Production of gibberellic acid by multiple fed-batch cultivation of *Gibberella fujikuroi*. **Chem. Biochem. Eng**, v. 21, n. 2, p. 159–162, 2007.

SHUKLA, R.; SRIVASTAVA, A.; CHAND, S. Bioprocess strategies and recovery processes in gibberellic acid fermentation. **Biotechnology and Bioprocess Engineering**, v. 8, p. 269–78, 2003.

SINGHANIA, R. R. et al. Recent advances in solid-state fermentation. **Biochemical Engineering Journal**, v. 44, n. 1, p. 13–18, abr. 2009.

SOCCOL, C. R.; VANDENBERGHE, L. P. . Overview of applied solid-state fermentation in Brazil. **Biochemical Engineering Journal**, v. 13, n. 2–3, p. 205–218, mar. 2003.

SOMOGYI, M. A new reagent for the determination of sugars. **The Journal of Biological Chemistry**, v. 160, p. 61–68, 1945.

SOWA, I. et al. Biofortification of soy (*Glycine max* (L.) Merr.) with strontium ions. **Journal of Agricultural and Food Chemistry**, v. 62, n. 23, p. 5248–5252, 2014.

STEFFENS, B.; WANG, J.; SAUTER, M. Interactions between ethylene, gibberellin and abscisic acid regulate emergence and growth rate of adventitious roots in deepwater rice. **Planta**, v. 223, n. 3, p. 604–612, 2006.

SUN, Y. et al. The potential of gibberellic acid 3 (GA 3) and Tween-80 induced phytoremediation of co-contamination of Cd and Benzo [a] pyrene (B [a] P) using *Tagetes patula*. **Journal of Environmental Management**, v. 114, p. 202–208, 2013.

TAIZ, L.; ZEIGER, E. **Fisiologia Vegetal**. 4ª ed. Porto Alegre: Artmed, 2009.

TANG, Z.; ZHOU, R.; DUAN, Z. Separation of gibberellic acid (GA3) by macroporous adsorption resins. **Journal of chemical Technology and Biotechnology**, v. 75, p. 695–700, 2000.

TANIMOTO, E. Tall or short? Slender or thick? A plant strategy for regulating elongation growth of roots by low concentrations of gibberellin. **Annals of botany**, v. 110, n. 2, p. 373–381, 2012.

TARKOWSKÁ D, NOVÁK O, FLOKOVÁ K, et al Quo vadis plant hormone analysis? **Planta** 240:55–76, 2014.

THAKARE, U.; PATIL, N.; MALPATHAK, N. Performance of chick pea under the influence of gibberellic acid and oxygenated peptone during germination. **Advances in Bioscience and Biotechnology**, v. 02, n. 01, p. 40–45, 2011.

TOMASINI, A.; FAJARDO, C. Gibberellic acid production using different solid-state fermentation systems. **World journal of microbiology & biotechnology**, v. 13, n. 5, p. 203–206, 1997.

TONON, R. V.; GROSSO, C. R. F.; HUBINGER, M. D. Influence of emulsion composition and inlet air temperature on the microencapsulation of flaxseed oil by spray drying. **Food Research International**, v. 44, n. 1, p. 282–289, 2011.

TUDZYNSKI, B. Biosynthesis of gibberellins in *Gibberella fujikuroi*: Biomolecular aspects. **Applied Microbiology and Biotechnology**, v. 52, n. 3, p. 298–310, 1999.

TUDZYNSKI, B. Gibberellin biosynthesis in fungi: genes, enzymes, evolution, and impact on biotechnology. **Applied microbiology and biotechnology**, v. 66, n. 6, p. 597–611, mar. 2005.

TUNA, A. L. et al. The combined effects of gibberellic acid and salinity on some antioxidant enzyme activities, plant growth parameters and nutritional status in maize plants. **Environmental and Experimental Botany**, v. 62, p. 1–9, jan. 2008.

URBANOVÁ, T. et al. Analysis of gibberellins as free acids by ultra performance liquid chromatography-tandem mass spectrometry. **Talanta**, v. 112, p. 85–94, 2013.

URBANOVA T, LEUBNER-METZGER G. Gibberellins and seed germination. In: The Gibberellins, Hedden P, Thomas SG (Eds). **Annual Plant Reviews.** Wiley-Blackwell Publishing Ltd., Oxford, UK, pp 253–284, 2016.

USLU, H. Distribution of Gibberellic Acid from the Aqueous Phase to the Organic Phase. **Journal of Chemical & Engineering Data**, v. 57, n. 3, p. 902–906, 2012.

USLU, H.; DATTA, D.; BAMUFLEH, H. S. Extraction Equilibria of Gibberellic Acid by Tridodecylamine Dissolved in Alcohols. **Journal of Chemical & Engineering Data**, v. 59, n. 11, p. 3882–3887, 2014.

UTHANDI, S.; KARTHIKEYAN, S.; SABARINATHAN, K. G. Gibberellic acid production by *Fusarium fujikuroi* SG2. **Journal of Scientific & Industrial Research**, v. 69, n. March, p. 211–214, 2010.

VASS, R.; JEFFERYS, E. Gibberellic acid. In: **Rose AH Economic microbiology**. 3. ed. London: Academic Press, 1979. p. 421–435.

WAHYUNI, S. et al. Improvement of Seedling Establishment of Wet Seeded Rice Using Ga 3 and Iba As Seed Treatment. **Control**, v. 4, n. 2, p. 56–62, 2003.

WANG, R. et al. Separation of gibberellic acid (GA3) by macroporous adsorption resins. **Frontiers of Chemical Engineering in China**, v. 2, n. 2, p. 171–175, 31 mar. 2008.

WANG, R.; TIAN, Z.; CHEN, L. A novel process for microencapsulation of fish oil with barley protein. **Food Research International**, v. 44, n. 9, p. 2735–2741, 2011.

WANG, Y. et al. Gibberellin in plant height control: old player, new story. **Plant Cell Rep** 36:391–398, 2017.

WANG, Y. et al. **Stable S-(+)- Abscisic acid liquid and soluble granule formulations**, US 8454982, 2013.

YAMAGUCHI, S. Gibberellin metabolism and its regulation. **Annual review of plant biology**, v. 59, p. 225–51, jan. 2008.

YARAŞ, A.; SELEN, V.; ÖZER, D. Synergistic effects of agro-industrial wastes on alpha amylase production by Bacillus amyloliquefaciens in semi solid substrate fermentation. **Pamukkale University Journal of Engineering Sciences**, v. 21, n. 7, p. 314–318, 2015.

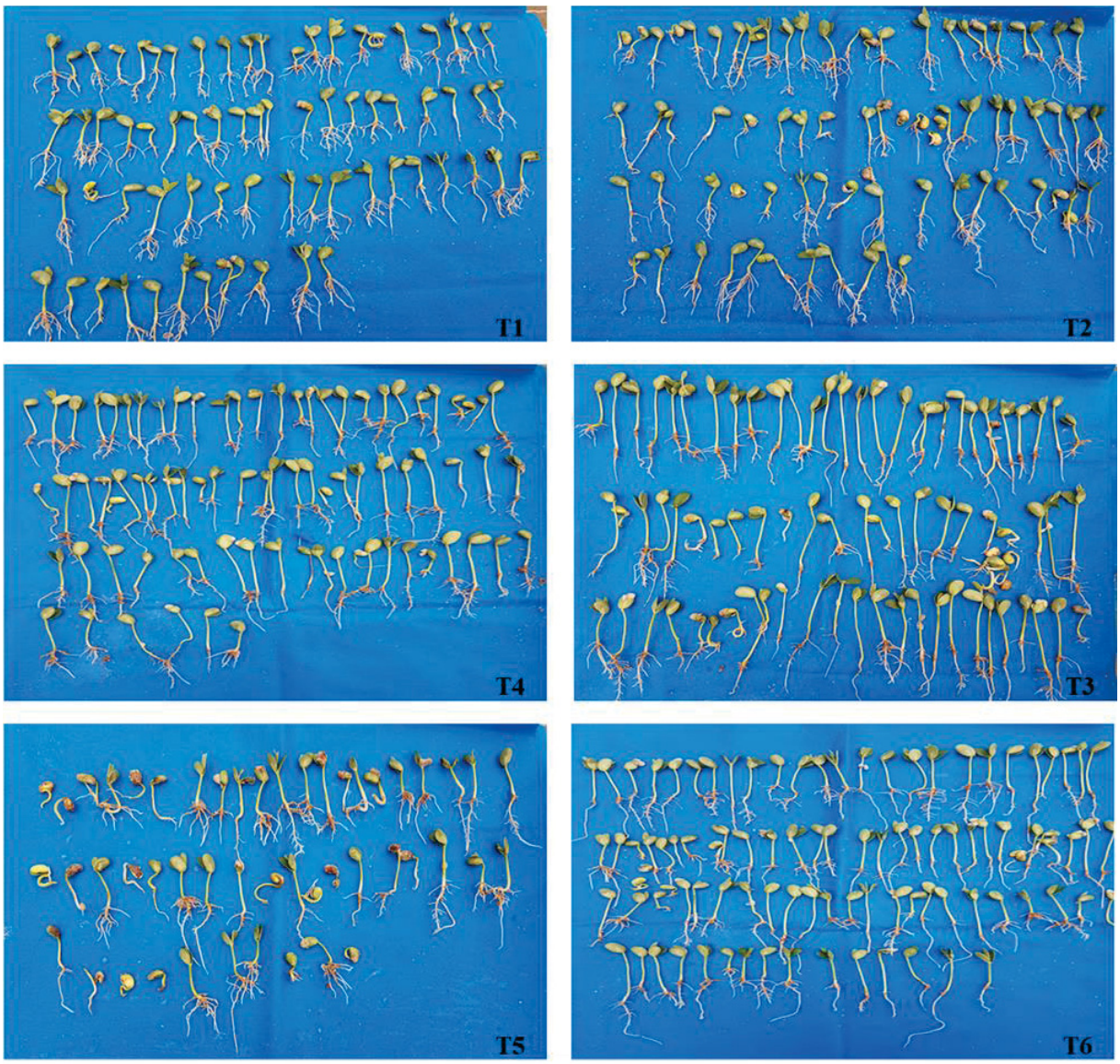
YOO, J. et al. Thermo-mechanical extrusion pretreatment for conversion of soybean hulls to fermentable sugars. **Bioresource Technology**, v. 102, n. 16, p. 7583–7590, 2011.

YULIA, E. et al. Resistance Potential to Powdery Mildew (Microsphaera diffusa Cooke and Peck) of Several Yellow and Black Soybean (*Glycine max* (L .) Merr) Genotypes. **KnE Life Sciences**, v. 2017, p. 270–278, 2017.

ZANG, Y. et al. Effect of gibberellic acid application on plant growth attributes , return bloom , and fruit quality of rabbiteye blueberry. **Scientia Horticulturae**, v. 200, p. 13–18, 2016.

ZHANG, F.; PAN, B.; SMITH, D. L. Application of gibberellic acid to the surface of soybean seed (*Glycine max* (L.) Merr.) and symbiotic nodulation, plant development, final grain and protein yield under short season conditions. **Plant and Soil**, v. 188, n. 2, p. 329–335, 1997.

ATTACHMENT A – EFFECT OF DIFFERENT TREATMENTS IN SOYBEAN SEEDS DEVELOPMENT AFTER 7 DAYS OF GERMINATION. T1- WITHOUT IMMERSION; T2- DEIONIZED WATER; T3- PROGIBB® 400; T4- GA_3 EXTRACT; T5- GIBBTEC LS; T6- GIBBTEC SP.



ATTACHMENT B – BOOK CHAPTER

In: Gibberellins and Gibberellic Acid
Editor: Jackson Hardy

ISBN: 978-1-63482-549-8

Chapter 1

GENERAL ASPECTS AND APPLICATIONS OF GIBBERELINS AND GIBBERELLIC ACID IN PLANTS

Marcela Candido Camara, Cristine Rodrigues [*],
André Luis Lopes da Silva,
Luciana Porto de Souza Vandenberghe
and Carlos Ricardo Soccol
Bioprocess Engineering and Biotechnology Department,
Federal University of Paraná, Curitiba, Paraná, Brazil

ABSTRACT

Gibberellins (GA) are a large group of tetracyclic diterpenoid carboxylic acids, which are essential for many processes in plants, including seed germination, stem elongation, sex determination, fruit set, senescence retardation, leaf expansion, trichome development, pollen maturation and induction of flowering. In combination with other hormones, it helps the plant to overcome the abiotic stress. Until now, more than 130 GAs have been identified in plants, fungi and bacteria, although only a few of them have biological activity. The major bioactive GAs includes GA_1, GA_3, GA_4 and GA_7, whose pronounced biological effects become commercially important. Most of the components of the GA signaling pathway in plants have been identified from genetic screens

[*] Corresponding author: Bioprocess Engineering and Biotechnology Department, UFPR, CEP 81531-980, Curitiba, Parana, Brazil. E-mail: cristinelabor@gmail.com.

ATTACHMENT C – CHAPTER 2 PUBLISHED ARTICLE

Planta
https://doi.org/10.1007/s00425-018-2959-x

REVIEW

Current advances in gibberellic acid (GA_3) production, patented technologies and potential applications

Marcela C. Camara[1] · Luciana P. S. Vandenberghe[1] · Cristine Rodrigues[1] · Juliana de Oliveira[1] · Craig Faulds[2] · Emmanuel Bertrand[2] · Carlos R. Soccol[1]

Received: 31 January 2018 / Accepted: 25 July 2018

Abstract

Main conclusion **Gibberellic acid is a plant growth hormone that promotes cell expansion and division. Studies have aimed at optimizing and reducing production costs, which could make its application economically viable for different cultivars.**

Gibberellins consist of a large family of plant growth hormones discovered in the 1930s, which are synthesized via the terpenes route from the geranylgeranyl diphosphate and feature a basic structure formed by an *ent*-gibberellane tetracyclic skeleton. Among them, only four have biological activity, including gibberellic acid (GA_3), which acts as a natural plant growth regulator, especially for stem elongation, seed germination, and increased fruit size. It can be obtained from plants, fungi, and bacteria. There are also some reports about microalgae GA_3 producers. Fungi, especially *Gibberella fujikuroi*, are preferred for GA_3 production via submerged fermentation or solid-state fermentation. Many factors may affect its production, some of which are related to the control and scale-up of fermentation parameters. Different GA_3 products are available on the market. They can be found in liquid or solid formulations containing only GA_3 or a mixture of other biological active gibberellins, which can be applied on a wide variety of cultivars, including crops and fruits. However, the product's cost still limits its large and continuous application. New low-cost and efficient GA_3 production alternatives are surely welcome. This review deals with the latest scientific and technological advances on production, recovery, formulation, and applications of this important plant growth hormone.

Keywords Plant growth regulators · *Fusarium fujikuroi* · Submerged fermentation · Alternative substrate · Downstream · Formulation

Introduction

Gibberellic acid (GA_3) is a diterpenoid carboxylic acid that belongs to the gibberellins family and acts as a natural plant growth hormone. Plants and some microorganisms, such as fungi and bacteria, produce it. GA_3 has promising applications in the agro-industrial sector due to its properties related to plant development. Since it was discovered, studies have been focused on enhancing its process yield, boosting its productivity, and reducing its cost, which sometimes restricts the use of this important growth hormone.

GA_3 is applied to crops, orchards, and ornamental plants, where it plays a role in seed germination (Finch-Savage and Leubner-Metzger 2006; Chen et al. 2008; Urbanova and Leubner-Metzger 2016), response to abiotic stress (Colebrook et al. 2014), fruit growth enhancement (Li et al. 2011), stem elongation (Dayan et al. 2012; Wang et al. 2017), flowering (Sharma and Singh 2009; Muñoz-Fambuena et al. 2012), the malting of barley (Briggs 1963), and other physi-

✉ Luciana P. S. Vandenberghe
lvandenberghe@ufpr.br

[1] Bioprocess Engineering and Biotechnology Department