Universidade Federal de Pernambuco
Centro de Ciências Biológicas
Programa de Pós-Graduação em Genética

**Luiz Rodrigo Saldanha Gazzaneo**

# EXPRESSÃO HETERÓLOGA DA DEFENSINA DEHYS DE *EUPHORBIA HYSSOPIFOLIA* EM *E. COLI*

**Recife**

**2016**

**Luiz Rodrigo Saldanha Gazzaneo**

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Tese apresentada ao Programa de Pós-Graduação em Genética da Universidade Federal de Pernambuco como parte dos requisitos exigidos para obtenção do título de Doutor em Genética sob a orientação do Prof. Dr. Antônio Carlos Freitas e coorientação da Dra. Valesca Pandolfi.

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"A única forma de chegar ao impossível, é acreditar que é possível."

Alice, no livro Alice País das Maravilhas
de Lewis Carroll - 1865

## RESUMO

Defensinas são peptídeos antimicrobianos (AMPs) que apresentam atividade contra diversos microrganismos patogênicos, em especial fungos. Embora não totalmente elucidados, há diversos mecanismos de ação propostos para as defensinas, que incluem permeabilização seletiva ou ruptura da membrana plasmática de microorganismos, ação direta em alvos intracelulares, ativação de cascatas de sinalização e aumento da produção de espécies reativas de oxigênio. Desde a sua descoberta e, tendo em vista sua ampla atividade biológica, o uso de defensinas no melhoramento de plantas cultivadas, bem como na produção de novos medicamentos tem sido proposto. Estudos de atividade biológica e possível aplicação biotecnológica das defensinas demandam uma grande quantidade dessas proteínas. Entretanto, o processo de extração da mesma é laborioso, dispendioso e, de acordo com a população ou disponibilidades da espécie vegetal escolhida, não sustentável ecologicamente. Portanto, a utilização de sistemas heterólogos de expressão é uma importante ferramenta para obtenção de defensinas recombinantes em escala industrial. Nesse estudo, um gene de defensina "*DeHys"*, isolado da *Euphorbia hyssopifolia,* foi inserido no plasmídeo pET102/D-TOPO e células da linhagem BL21(DE3) de *Escherichia coli* foram transformada com essa construção. Foi produzida a defensina recombinante Dehys com tamanho aproximado de 24 kDa. Sua identidade foi confirmada por western blot e pela análise do padrão de digestão com proteases.



**Palavras-chave**: Peptídeo antimicrobiano. Defensina. *E.coli*. Proteína recombinante.

**ABSTRACT**

Defensins are antimicrobial peptides (AMPs) , which present activity against a variety of pathogenic microorganism, especially funghi. Although not completely elucidated, there are a variety of proposed mechanisms of action for defensins, which includes selective microorganisms plasmatic membrane permeabilization or rupture, straight action agains intracellular targets, activation of signaling cascades and the burst of reactive oxygen species. Since it's discovery and due to it's wide biological activities, it's use in crop enhancing, as well as its use in the development of new drugs have been proposed. Defensin's biological activity and biotecnological application studies demand a reasonable amount of purified protein. However, the extraction processes is laborious, expensive, time consuming and depending on the population or the chosen plant species supply, not ecologically sustainable. So, the use of heterologous expression sytems is an importante tool to obtain purified proteins in industrial scale. In this study, a defensing gene (*Dehys*) isolated from *Euphorbia hyssopifolia was inserted in a p* pET102/D-TOPO vector and trasnformed into BL21(DE3) *Escherichia coli* strains. A recombinat Dehys defensin of approximately 24 kDa was obtained. It's identity was double-checked using by Western blot and protease digestion pattern analyses.

**Key words:** Antimicrobial peptides. Defensin. *E. coli*. Recombinant protein.

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## LISTA DE TABELAS

# LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

| | |
|---|---|
| AMP | **Antimicrobial peptide / Peptídeo antimicrobiano** |
| APD2 | **Antimicrobial Peptide Database / Banco de Dados de Peptídeos Antimicrobianos** |
| cDNA | **DNA complementar** |
| CIs | **Corpos de inclusão** |
| CSαβ | **Cysteine-Stabilized αβ motif / Motivo αβ Estabilizado por Cisteínas** |
| dN | **Non-synonymous substitutions / Substituições não-sinônimas** |
| dS | **Synonymous substitutions /Substituições sinônimas** |
| DTT | **Dithiothreitol** |
| *E. coli* | **Escherichia coli** |
| ePC | **Fosfatidilcolina do ovo** |
| gor | **Glutathione redutase** |
| GST | **Glutathione-S-transferase** |
| HES | **Heterologous expression system / Sistema de Expressão Heteróloga** |
| IB | **Inclusion body / corpo de inclusão** |
| IC50 | **Inhibitory concentration of a drug to decrease a biological process by half / Concentração inibitória de uma droga que diminui um processo biológico pela metade.** |
| IPTG | **Isopropyl b-D-1-thiogalactopyranoside** |
| LB medium | **Luria-Bertani Medium** |
| PVDF | **Polyvinylidene difluoride / Polivinilideno difluorido** |
| RNAt | **RNA transportador** |
| TRX | **Thioredoxina** |
| trxB | **Thioredoxina redutase** |

**SUMÁRIO**

## 1 INTRODUÇÃO

As primeiras defensinas vegetais foram descobertas em 1990, por Mendez e colaboradores em sementes de cevada e trigo. Inicialmente foram chamadas de δ-tioninas, devido a sua similaridade em tamanho e número de pontes dissulfídicas com as α-tioninas e β-tioninas. Defensinas são pequenas proteínas (<10 Kda), altamente básica, ricas em cisteínas e participam do sistema imune inato como uma estratégia de secreção de proteínas contra microrganismos invasores. Defensinas exibem uma variedade de atividades biológicas, especialmente atividade antifúngica e antibacteriana. Em plantas apresentam comprimento que varia de 45 a 54 aminoácidos.

Desde a sua descoberta, estudos relacionados com as rotas de ativação de defensinas, modo de ação, e potencial para o aumento de resistência contra fitopatógenos tem sido desenvolvidos. O fato de defensinas vegetais serem destituídas de toxicidade contra células de mamíferos tem aberto um novo horizonte de pesquisas: o seu potencial terapêutico contra doenças infecciosas humanas.

Isolar defensinas de sua fonte natural, como tecido vegetal, pode consumir muito tempo, ser trabalhoso e normalmente apresentam um baixo rendimento. Portanto, outras estratégias para a produção em larga escala de quantidades satisfatórias tem sido empregadas através da tecnologia do DNA recombinante. Um número crescente de estudos tem reportado o uso de diferentes sistemas de expressão heteróloga (SEH) para a produção de defensinas. Contudo, como toda tecnologia, SEHs também apresentam limitações práticas e pontos a serem observados, tanto em sistemas baseados em bactérias, fungos ou plantas, demandando um desenho experimental cuidadoso.

Nesse contexto, nosso trabalho visou construir um vetor de expressão contendo um minigene otimizado para uma defensina de Euphorbia hyssopifolia, cuja sequência foi obtida pelo Laboratório de Genéticas e Biotecnologia Vegetal – LGBV, e ,através do uso de um sistema de expressão heteróloga baseado em Escherichia coli, produzir a defensina recombinante para futuro estudos de atividade biológica.

# 2 REVISÃO

## 2.1 PEPTÍDEOS ANTIMICROBIANOS (AMPS)

Há tempos a idéia de que apenas os vertebrados apresentam mecanismos específicos de defesa contra invasores microbianos foi modificada devido a estudos que comprovaram que plantas também apresentavam sistemas para o reconhecimento e eliminação específica do não-próprio (GARCÍA-OLMEDO et al., 1998).

Uma estratégia de defesa muito antiga e amplamente distribuída entre os seres vivos multicelulares é a produção e exsudação de pequenos peptídeos com atividade antimicrobiana, denominados “peptídeos antimicrobianos” (AMPs). O tamanho reduzido e o fato de serem codificados por genes únicos, tornam a síntese dessas moléculas algo rápido, versátil, e de baixo investimento energético ou metabólico (BROEKAERT et al., 1995; THOMMA et al., 2001).

AMP’s apresentam diversas atividades biológicas, atuando tanto na defesa contra infecções por patógenos, como leveduras, bactérias e vírus (FRANCO et al., 2006; THEVISSEN et al., 2004; ZOUBENKO el at., 1997), protozoários e helmintos (BORENSTEIN et al., 1991; COLGRAVE et al., 2009). Herbívoros invertebrados também sofrem o efeito desses peptídeos, como pode ser observado em plantas, que por não apresentarem mobilidade, não podem fugir de seus predadores, em contrapartida, conseguem inibir a perda de sua biomassa através da produção desses peptídeos, com ação inseticida (JENNINGS et al. 2001), moluscicida (PLAN et al., 2008) ou mesmo inibindo a ação de importantes enzimas hidrolíticas, como a $\alpha$-amilase, envolvidas na digestão do seu material vegetal (FRANCO et al., 2002).

Apesar de sua ampla diversidade, os AMPs apresentam características em comum; como carga geral positiva, resistência a ação de solventes ácidos e orgânicos, ampla atividade biológica e estabilidade térmica. Além disso, a análise da composição de aminoácidos e estruturas secundária e terciária dos AMPs permite classificá-los em peptídeos aniônicos, peptídeos α-hélice catiônicos lineares, peptídeos catiônicos enriquecidos com aminoácidos específicos, peptídeos aniônicos e catiônicos formados por fragmentação de peptídeos maiores, e por fim, peptídeos aniônicos e catiônicos que contêm cisteína e apresentam pontes dissulfídicas (BROGDEN, 2005).

## 2.2 DEFENSINAS

As defensinas (peptídeos anti-infectivos) constituem uma superfamília dentre os AMPs, presentes nas mais variadas formas de vida: plantas, mamíferos (THOMMA; CAMMUE; THEVISSEN, 2002), pássaros, moluscos (CHARLET et al., 1996), insetos, aracnídeos (COCIANCICH et al., 1993) e fungos (OARD; KARKI, 2006).Composta por peptídeos com estrutura e função altamente conservadas entre vertebrados, invertebrados e vegetais, as defensinas tem como características em comum a predominância de resíduos de cisteína, formação de pontes dissulfídicas e presença de folhas-β antiparalelas estáveis (CASTRO; FONTES, 2005).

As primeiras defensinas vegetais descobertas por Mendez et al. em 1990 quando foram inicialmente denominadas de c-tioninas devido a sua semelhança com as tioninas já descritas e pelo seu conteúdo de cisteínas. Porém, estudos posteriores demostraram a presença de diferenças entre as duas classes de moléculas, em especial no padrão de pontes dissulfíticas apresentado pelas defensinas (até então c-tioninas) (BRUIX et al., 1995).

Além do caráter extremamente básico dado pela sua composição de aminoácidos e abundância de cisteínas, as defensinas vegetais são peptídeos pequenos com comprimento variando entre 45 e 54 aminoácidos, cuja cadeia encontra-se estabilizada por 4 pontes dissulfídicas (ALMEIDA et al., 2002).

## 2.3 MECANISMOS DE AÇÃO

Os mecanismos de ação das defensinas (peptídeos catiônicos), embora não totalmente elucidados, consiste, basicamente, no aumento da permeabilidade da membrana das células atacadas, devido a sua afinidade e inserção nas mesmas, formando canais iônicos que alteram o potencial da membrana plasmática devido ao influxo de $Ca^{2+}$ e do efluxo de $K^{+}$ (SUGIARTO; YU, 2004). Estudos demonstraram que além de atuar na membrana plasmática, algumas defensinas podem ter alvos intracelulares, como é o caso da defensina de *Pisum sativum* (Psd1) e a ciclina F encontrada no núcleo de células fúngicas (LOBO et al., 2007). Sua interação leva a uma interferência no ciclo celular, o que poderia ser explorado no tratamento de doenças causadas por mitoses excessivas como o câncer.

Um trabalho recente usando a defensina vegetal PpDFN1 demonstrou que a adição do esfingolipídeo ceramida neutro β-D-galactosídeo a uma monocamada

lipídica de fosfatidilcolina de ovo (ePC) aumentou a afinidade da defensina à membrana artificial. Ainda, quando o mesmo experimento foi realizado usando lipídeos isolados de fungos fitopatogênicos, a afinidade apresentada foi ainda maior, demonstrando que a ligação de uma defensina vegetal a uma membrana-alvo de um patógeno é dependente da sua concentração e composição lipídica (NANNI et al., 2013). O mesmo estudo também demonstra, por teste de hemólise, não haver atividade da PpDFN1 contra eritrócitos humanos, em consonância com os achados de Wong e Ng (2005) em vulgarina, uma defensina isolada de *Phaseolus vulgaris*. Esta última não apresentava toxicidade contra células do baço de mamíferos e eritrócitos. Novamente, em Nanni e colaboradores (2013), assim como em Golçalves e colaboradores (2012) as defensinas vegetais estudadas não apresentaram afinidade à bicamadas lipídicas enriquecidas com colesterol, bicamada essa similar às membranas de células de mamíferos.

## 2.4 FUNÇÕES BIOLÓGICAS

Sementes são órgãos de armazenamento de energia, tornando-se alvo para uma variedade de organismos heterotróficos, especialmente fungos. Defensinas desempenham um papel *in vivo* de proteção de sementes durante a germinação criando um microambiente ao redor das sementes onde o crescimento de fungos é suprimido (TERRAS et al., 1995).

Outro papel importante é o de proteção durante a formação do embrião com a expressão local da defensina logo após a polinização (BALANDIN et al., 2005); além de proteção contra estresses bióticos, que incluem fungos (AHMAD et al., 2011), bactérias (MAAROF et al., 2011), insetos herbívoros (ABE et al., 2008), nematóides (SIDDIQUE et al., 2011) e plantas parasíticas (LETOUSEY et al., 2007). Apresentam também uma função nas interações simbióticas, sendo negativamente reguladas nas raízes quando na presença de bactérias fixadoras de nitrogênio (JOHANSSON et al., 2004).

## 2.5 EXPRESSÃO HETERÓLOGA EM *E. COLI*

A produção de proteínas altamente purificadas e bem caracterizadas tem se tornado um dos principais objetivos da indústria farmacêutica (SCHMIDT, 2004). Sistemas de expressão heteróloga baseados em bactérias apresentam atrativos

como o rápido ganho de biomassa em substratos mais baratos, a disponibilidade de uma grande variedade de vetores e linhagens muito bem caracterizadas geneticamente (TERPE, 2006). A bactéria mais utilizada para produção de proteínas é a *Escherichia coli*, porém proteínas para uso terapêutico devem passar por uma segunda etapa de purificação para garantir a retirada de endotoxinas, lipopolissacarídeos pirogênicos em humanos e outros mamíferos (PETSCH; ANSPASH, 2000). A produção citoplasmática é a mais frequente por apresentar um maior rendimento, porém já existem linhagens capazes de secretar a proteína recombinante em grande quantidade (GEORGIOU; SEGATORI, 2005). Uma desvantagem natural dessas bactérias seria a incapacidade de realizar processamentos pós-traducionais no seu citoplasma, como a formação de pontes dissulfídicas, devido ao ambiente altamente redutor. Por essa razão, é comum trabalhos onde as proteínas recombinantes são direcionadas para o periplasma, no qual proteínas catalizadoras de pontes dissulfídicas (DsbA, DsbB, DsbC, DsbD), e peptidil-prolil isomerases (SurA, RotA, Fk1B, and FkpA) (JOLY; SWARTZ, 1994; SHOKRI et al., 2003) irão auxiliar neste tipo de processamento.

A expressão citoplasmática com “dobramento” correto de defensinas em *E. coli*. é possível através do uso de linhagens mutantes para trxB tioredoxina redutase e ainda, se isomerares, como a enzima DsbC (também chamada de foldase) tiverem sido engenheiradas para serem também expressas no citoplasma (SANTOS et al., 2010). Algumas linhagens comerciais apresentam plasmídeos codificando RNAt raros, atenuando assim o “viés do códon”, tornando a otimimização de códon menos crucial para o sucesso da expressão (HUANG; REUSCH, 1995). No mercado é possível adquirir linhagens que acumulam essas duas modificações, levando a um melhor controle da expressão e formação de ligações dissulfídicas no citoplasma em proteínas heterólogas produzias por *E. coli* (BOTTCHER et al. 2007).

## 2.6 PROSPECÇÃO DE DEFENSINAS DE PLANTAS

A obtenção de uma sequência do peptídeo maduro de uma defensina de planta pode ser feita basicamente por duas estratégias principais: genética reversa ou prospecção em bancos de dados. A estratégia de genética reversa consiste basicamente na extração e purificação de defensinas vegetais nativas a partir de tecidos, seguida por sequencimento de aminoácidos. A prospecção de sequências

de defensina (data mining) baseia-se na utilização de ferramentas de bioinformática para encontrar sequências com motivos semelhantes a defensinas em bancos de dados genômicos de plantas. Em ambos os casos, o conhecimento da sequência de defensina irá fornecer a informação necessária para o desenho de primers para a amplificação de cDNA e/ou plasmídeos artificiais contendo a sequência de defensina madura.

## 2.7 GENÉTICA REVERSA

A estrutura conservada e características bioquímicas das defensinas vegetais permitem a purificação de peptídeos previamente desconhecidas a partir de plantas para as quais as sequências do genoma ainda não estão disponíveis. Embora as sementes sejam o tecido mais utilizado para o isolamento de defensinas (FINKINA et al., 2008; GAMES et al., 2008; PELEGRINE et al., 2008), as defensinas também podem ser isoladas a partir de outras partes da planta. Lay et al. (2003) isolaram uma defensina em tabaco ornamental e duas em petúnia, em ambos os casos, usando tecido floral. Através da análise de immunoblot, eles descobriram que as defensinas foram mais concentradas nos ovários, pétalas e pistilos durante as fases iniciais de desenvolvimento da flor. O isolamento de defensinas também foi relatado em mesocarpo de frutos maduros de Capsicum annuum (MAAROF et al., 2011), em raízes tuberosas de Ipomoea batatas (L.) Lam. 'Tainong 57' (HUANG et al., 2008), em ferimentos de Arabidopsis thaliana (PENNINCKX et al., 1996) e também em folhas intactas de Spinacia oleracea (SEGURA et al., 1998)

O processo de extração muitas vezes consiste em pulverizar o tecido, produzindo um pó de semente ou maceramento de tecidos moles com nitrogênio líquido, seguido da adição de um tampão salino e de extração a baixa temperatura (4° C). O sobrenadante é então recolhido e exposto a diferentes concentrações de sulfato de amônio (CARVALHO et al., 2001) causando a precipitação de grupos de proteínas com base nas suas cargas. O precipitado para cada concentração de sulfato de amônio é então ressuspenso e submetido a métodos cromatográficos, a fim de se obter uma amostra purificada do peptídeo alvo (GAMES et al., 2008). Devido ao seu perfil catiônico, a amostra passa através de uma coluna de cromatografia de afinidade (coluna de fraca permuta aniônica) e a fração contendo as defensinas é então aplicada a uma coluna de cromatografia de exclusão por

tamanho (um processo também referida como cromatografia de filtração em gel) (Wang, 2002).

## 2.8 ESTRATÉGIA DE PROSPECÇÃO DE BANCOS DE DADOS

A crescente resistência de agentes patogénicos microbianos aos fármacos criou a necessidade da descoberta de novas drogas anti-microbianos. Peptídeos antimicrobianos, especialmente defensinas vegetais, têm provado serem eficazes contra muitos patógenos bacterianos e fúngicos. Portanto, o desenvolvimento de bancos de dados de AMPs e defensinas tem ajudado muitos pesquisadores em seus estudos, proporcionando não apenas as sequências, mas ferramentas para melhorar a forma como a informação é apresentada, levando a uma melhor compreensão de sua proteína de interesse. Alguns bancos de dados disponibilizam programas para executar buscas por homologia, alinhamento múltiplo de sequências, filogenia, perfis físico-química além de outros cálculos e previsões (HAMMAMI; FLISS, 2010)

Um exemplo de banco de dados é o PhyAMP - um repositório específico com dados de peptídeos antimicrobianos naturais de plantas. Registos de 55 defensinas de plantas podem ser encontrados no seu banco de dados, juntamente com diagramas e gráficos muito úteis sobre a árvore filogenética dessas defensinas, distribuição de aminoácidos básicos e ácidos, e atividade. Ferramentas que tornam a prospecção mais fácil ou que fornecem novos dados para os estudos, como busca por similaridade, Modelo Oculto de Markov, perfil físico-químico e alinhamento de sequências estão disponíveis no PhyAMP (HAMMAMI et al., 2009). Em alguns casos, se um pesquisador está familiarizado com a proteína que ele está procurando, alguns bancos de dados não-específicos, tais como GenBank (www.ncbi.nlm.nih.gov/genbank), também pode ser úteis (KARRI; BHARADWAJA, 2013)

Como um exemplo, Karri e Bharadwaja (2013) procuraram as sequências de Tfgd2 (Trigonella foenum - graecum defensina 2; GenBank número de acesso AY227192) e RsAFP2 (Raphanus sativus proteína antifúngica 2; número de acesso GenBank U18556) no GenBank para construir um gene de fusão Tfgd2-RsAFP2 (que mais tarde foi depositado em GenBank com o número de acesso KF498667). A construção Tfgd2-RaAFP2 provou ser três vezes mais eficiente contra a germinação

de conídeos de *Phaeoisariopsis personata*. Uma lista contendo alguns dos bancos de dados de AMPs e defensinas disponíveis é fornecida na Tabela 1.

Tabela 1. Banco de dados de peptídeos antimicrobianos e defensinas.

| Banco de Dados | Ano* | Descrição | URL |
|---|---|---|---|
| **LAMP** | 2013 | "LAMP: a Database Linking Antimicrobial Peptides". | http://biotechlab.fudan.edu.cn/database/lamp |
| **DAMPD** | 2011 | DAMPD: uma atualização e substitute ao banco de dados ANTIMIC. | http://apps.sanbi.ac.za/dampd |
| **CAMP** | 2009 | "Collection of Antimicrobial Peptides", India. | http://www.bicnirrh.res.in/antimicrobial |
| **RAPD** | 2008/09 | Um banco de dados de peptídeos antimicrobianos recombinants. Estados Unidos da América. | http://faculty.ist.unomaha.edu/chen/rapd/ |
| **APD2** | 2004/09 | "The Antimicrobial Peptide Database". Estados Unidos da América. | http://aps.unmc.edu/AP/main.html |
| **PhyAMP** | 2008 | Um banco de dados dedicado a peptídeos antimicrobianos vegetais. Tunisia. | http://phytamp.pfba-lab-tun.org/ |
| **Defensins** | 2007 | Conhecimentos sobre defensinas. Singapura. | http://defensins.bii.a-star.edu.sg/ |
| **AMPer** | 2007 | Um banco de dados e ferramenta de busca para peptídeos antimicrobianos, baseada em modelos oculto de Markov e no banco de dados da SwissProt. Canada. | http://marray.cmdr.ubc.ca/cgi-bin/amp.pl |
| **AMSDb** | 2002/04 | "Antimicrobial sequence Database". Italy. | http://www.bbcm.univ.trieste.it/~tossi/amsdb.html |

* Data de criação e/ou atualização do banco de dados.

## 2.9 EXPRESSÃO HETERÓLOGA DE DEFENSINAS VEGETAIS

Avanços na tecnologia do DNA recombinante têm fornecido uma oportunidade para a produção de altos níveis de defensinas vegetais. Esta tecnologia permite a clonagem de genes estranhos em vectores específicos para a expressão em procariotas e/ou sistemas eucarióticos, e é considerado o método mais eficaz levando-se em consideração o tempo e custos de produção (XU et al., 2007). As proteínas têm várias características que devem ser cuidadosamente observados durante a escolha de um sistema para a sua produção heteróloga, tais como tamanho, localização intracelular ou secreção, dobragem correcta e padrão de glicosilação (DESAI; SHRIVASTAVA; PADH, 2010).

Os principais hospedeiros utilizados para a produção AMPs são bactérias e leveduras, que representam 97,4% das AMPs expressa-heterólogas (DESAI; SHRIVASTAVA; PADH, 2010; LI: CHEN, 2008). Mais recentemente, as plantas têm emergido como um promissor hospedeiro para a produção de AMP já que plantas transgénicas podem ser usadas diretamente no controlo microbiano, expressando o peptídeo no cultivar desejado (DESAI; SHRIVASTAVA; PADH, 2010; GIDDINGS et al., 2000).

Diferentes hospedeiros têm sido utilizados para a produção de defensinas vegetais em sistemas procarióticos e eucarióticos. Defensinas vegetais já foram produzidos em bactérias (NANNI et al., 2013; SOLIS; MEDRANO; GHISLAIN, 2007; SUDAR; KIRTI, 2006), leveduras (CABRAL et al., 2003; CHEN et al., 2004; KANT; LIU; PAULS, 2009) e plantas transgênicas (ABDALLAH et al., 2010; CHOI et al., 2009; NTUI et al., 2010) com graus variáveis de sucesso e produção de proteína.

## 2.10 SISTEMA DE EXPRESSÃO DE *ESCHERICHIA COLI*

A *Escherichia coli* é o microrganismo mais utilizado para a produção de proteína heteróloga. Este sistema é a primeira escolha para a produção de defensinas vegetais e até o momento apresenta os maiores rendimentos relatados. Há muitas razões para isso: *E. coli* tem provado ser o método mais rentável de produção de proteínas recombinantes devido ao seu rápido crescimento, grande disponibilidade de vetores de expressão comercial, protocolos de manipulação de DNA bem estabelecidos e amplo conhecimento sobre sua genética, bioquímica e fisiologia (SORENSEN; MORTENSEN, 2005). No entanto, existem algumas

desvantagens que devem ser superados a fim de alcançar uma produção eficiente em bactérias.

Defensinas vegetais já foram produzidos de forma heteróloga em sistemas de expressão de *E. coli.* Nanni et al. (2013) alcançou um rendimento de 0,5 mg L-1 dos PpDFN1 clonados utilizando o vector pET-32 (Novagen) e células (DE3) pLys Origami E. coli BL21 (Novagen). A defensina produzido era funcional e apresentou actividade antifúngica contra *Botrytis cinerea*, *Monilinia laxa* e *Penicillium expansum*, com valores de IC50 de 15,1, 9,9 e 1,1 µg.mL-1, respectivamente.

Da mesma forma Kovaleva et ai. (2011) já havia expressado a defensina *Pinus silvestres* 1 (PsDef1) no sistema PET (pET42a) e linhagem BL21 (DE3) de *E. coli*, embora ligada a uma glutationa-S-transferase como proteína de fusão (GST). Eles foram capazes de produzir uma defensina completa, que não era biologicamente activa quando fundida a GST, mas altamente funcional contra um uma gama de fungos patogênicos, quando separado da proteína de fusão.

A falta de estudos comparativos de defensinas planta recombinante expressas em diferentes linhagens torna difícil prever qual delas irá propiciar melhores resultados. Escolher o melhor sistema de expressão pode ser crucial para alcançar um rendimento satisfatório de uma defensina recombinante. Às vezes, um ensaio preliminar usando diferentes linhagens podem ajudar a fazer tal decisão, já que a produção de proteína recombinante pode variar muito entre linhagens diferentes (ZHANG et al., 2010).

Defensinas apresentam oito resíduos de cisteína que interagem criando quatro ligações dissulfídicas, por conseguinte, é importante que a linhagem de expressão escolhida seja capaz de formar tais dobra. A formação da ligação de dissulfídica é inibida no citoplasma de *E. coli*, por conta da presença de tiorredoxinas, e é dependente de uma enzima fosfatase alcalina, que é ativa apenas no periplasma (BOSSETTE et al., 1999). A expressão citoplasmática e dobragem correta das defensinas em *E. coli* tornou-se possível através do uso de linhagem mutantes para a tioredoxina redutase (trxB), e se isomerases periplasmáticas, tais como a isomerade para ligações dissulfídicas (DsbC, catalisa a formação e correção de ligações dissulfídicas, também chamado de foldases) forem manipuladas para serem expressas no citoplasma (QIU; SWARTZ; GEORGIOU, 1998). Algumas linhagens apresentam plasmídeos que codificam RNAt’s raros atenuando o viés dos códons, tornando a otimização de códons menos crucial para uma expressão bem

sucedida (HUANG; REUSCH, 1995); outros fornecem um ambiente mais oxidado no citoplasma aumentando a formação de ligações dissulfídicas nas defensina recombinante, devido a mutações nos genes trxB e glutationa redutase (GOR) (SANTOS et al., 2010). Há também linhagens disponíveis no mercado que combinam estas duas modificações, para um melhor controle da expressão e formação citoplasmática de ligações dissulfídicas nas proteínas heterólogas expressas em *E. coli* (BOTTCHER et al., 2007).

A degradação das proteínas durante os passos de purificação pode ser minimizada quando utilizando-se linhagens mutantes para a Ion protease e a protease de membrana externa (ompT) (ZHANG et al., 2010). É importante salientar que, mesmo com o desenho experimental robusto não é possível prever o resultado de uma produção defensina recombinante solúvel. Kovalskaya e Hammod (2009) construíram cassetes utilizando o vector de expressão pET26b e dois AMP's vegetais, uma snakin (SN1) e uma defensina (PTH1), para a produção heteróloga em linhagem BL21 (DE3). A estratégia para produzir moléculas solúveis falhou e as proteínas recombinantes foram encontradas concentradas em corpos de inclusão.

Há uma infinidade de linhagens para expressão disponíveis, cada uma com combinações diferentes de modificações estratégicas: mutantes para RNAse E para uma maior vida útil do RNA intracelular, T7 lisozima para um melhor controle de peptídeos tóxicos, promotores induzidos por sais para uma melhor solubilidade, mutantes lacY para uma expressão homogênea e expressão precisa, e adição das chaperonas DsbC para melhor "folding" citoplasmático (Tabela 2).

Tabela 2. Linhagens de *Escherichia coli* usdas para a expressão heteróloga de defensinas vegetais.

| **Bacterial Strain** | **Features** | **Manufacturer** |
|---|---|---|
| **BL21 (DE3)** | High Transformation efficiency, IPTG induction, deficient of Lon and OmpT proteases. Suitable for non-toxic products expression. | Novagen/ Stratagene |
| **BL21 (DE3)-pLysS** | Same as BL21 (DE3) with the addition of a plasmid, pLysS, which lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following IPTG induction. It is therefore suitable for expression of toxic genes. | Novagen/ Stratagene |
| **Origami 2** | Origami™ 2 host strains are K-12 derivatives that have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, which greatly enhance disulfide bond formation in the *E. coli* cytoplasm and are recommended only for the expression of proteins that require disulfide bond formation for proper folding. | Novagen |
| **Origami B** | Origami B host strains carry the same trxB/gor mutations as the original Origami strains, except that they are derived from a lacZY mutant of BL21. | Novagen |
| **Origami B (DE3)pLysS** | Same as Origami B with the addition of a plasmid, pLysS, which lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. Thus it is suitable for expression of toxic genes. | Novagen |
| **Rosetta (DE3)** | Rosetta host strains are BL21 lacZY (Tuner) derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA on a compatible chloramphenicol resistant plasmid. The tRNA genes are driven by their native promoters. | Novagen |
| **Rosetta (DE3)pLysS** | In Rosetta (DE3)-pLysS, the rare tRNA genes are presented on the same plasmids that carry the T7 lysozyme. | Novagen |
| **Rosetta-gami (DE3)LysS** | Rosetta-gami host strains are Origami derivatives that combine the enhanced disulfide bond formation resulting from trxB/gor mutations with enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli.* These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, GGA on a compatible chloramphenicol-resistant plasmid. The tRNA genes are driven by their native promoters. In Rosetta-gami(DE3)-pLysS, the rare tRNA genes are presented on the same plasmids that carry the T7 lysozyme. | Novagen |
| **BL21 CodonPlus** | BL21-CodonPlus-RIL chemically competent cells carry extra copies of the argU, ileY, and leuW tRNA genes. The tRNAs encoded by these genes recognize the AGA/AGG (arginine), AUA (isoleucine), and CUA (leucine) codons, respectively. | Stratagene |
| **AD494** | AD494 strains are thioredoxin reductase (trxB) mutants of the K12 strain that enable disulfide bond formation in the cytoplasm, providing the potential to produce properly folded active proteins. | Novagen |
| **BL21trxB** | BL21trxB strains possess the same thioredoxin reductase mutation (trxB) as the AD494 strains in the protease deficient BL21 background. The trxB mutation enables cytoplasmic disulfide bond formation. | Novagen |

## 2.11 VETORES

É importante destacar que se deve tentar combinar esforços e estratégias de vetores de expressão para tornar a expressão mais propensas ao sucesso. Por exemplo, a utilização de vectores de expressão carregando um sinal *pelB* N-terminal é recomendada quando se trabalha com linhagens mutantes para a protease lon e ompT, proporcionando uma localização periplásmica para a proteína recombinante, para melhores condições de dobragem e de solubilização (VIJAYAN; GURUPRASAD; KIRTI, 2008).

Os vectores de expressão podem adicionar moléculas de ligação para a proteína recombinante, tornando a purificação de proteínas um processo menos demorado e trabalhoso. Os vectores de expressão pMAL (comercializado por New England Biolabs), por exemplo, estão divididos em dois grupos principais: a pMAL-c e os vectores pMAL-p. Ambos os grupos adicionam uma proteína de ligação à maltose à defensina recombinante, fazendo com que o processo de purificação apresente um só passo. Os vectores pMAL-c (como o pMAL-c2x, pMAL-c5x, etc.) apresentam a deleção da sequência para o sinal *malE* resultando em uma expressão citoplásmica da proteína de fusão. Isso leva a melhores rendimentos (níveis de proteína recombinante de 20-40% da proteína celular total), mas isto não é recomendado para os peptídeos que necessitam de formação de ligações dissulfídicas (MAAROF et al., 2011). A série pMAL-p apresenta a sequência do gene para o sinal *malE* intacta, o que leva à secreção do péptido recombinante para o periplasma. A série pMAL-p apresenta os rendimentos mais baixos (os níveis de proteína recombinante de 1-20% da proteína celular total), mas é mais propenso a formar a dobras corretas nas ligações dissulfídicas.

Para expressão heteróloga de defensinas vegetais, o sistema de expressão mais amplamente utilizado é o sistema pET, desenvolvida inicialmente por W. F. Studier e B. A. Moffatt, em 1968, que criou um sistema de expressão de RNA polimerase que era altamente selectivo para a RNA polimerase do bacteriófago T7 (STUDIER; MOFFATT, 1986). Estes vectores têm como característica principal um forte sinal de transcrição do bacteriófago, conduzindo a um elevado nível de expressão heteróloga, com a proteína recombinante que compreende até 50% das proteínas celulares totais em apenas algumas horas após a indução. Estes vectores devem ser clonado em linhagens que possuam o gene da T7 polimerase para

expressão. Linhagens, como BL21 (DE3) foram modificadas para apresentar o gene da T7 polimerase sob o controle de um promotor *lac* derivado L8-UV5 (PAN; MALCOLM, 2000). O promotor derivado do *lac* L8-UV5 contém três mutações pontuais, que aumentam a força do promotor, diminuem a sua dependência do AMP cíclico e cria um promotor mais forte que é menos sensível à glicose. Estas modificações permitem uma forte indução da T7 RNA polimerase utilizando IPTG como indutor (GROSSMAN, 1998).

## 2.12 PROTEÍNAS CARREADORAS E SINAIS DE SECREÇÃO

Não é surpreendente que expressar um péptido antimicrobiano num sistema procariótico podem conduzir à morte das células hospedeiras. A utilização de uma proteína carreadora ajuda a minimizar os efeitos tóxicos das defensinas expressas nas células hospedeiras. Devido ao seu tamanho reduzido, as defensinas são propensas a ser degradada por proteases intracelulares. Tal degradação pode ser superado pela fusão a uma proteína carreadora ao peptídeo heterólogo. A proteína carreadora imita os pró-segmentos de proteínas nativas de *E. coli* protegendo a proteína fundida de ataques por proteases intracelulares.

As duas proteínas carreadoras mais frequentemente usadas para a expressão solúvel em *E. coli* são a tioredoxina (TRX) e a glutationa-S-transferase (GST). Bogomolovas et ai. (BOGOMOLOVAS, et al., 2009) demonstraram que entre 13 parceiros de fusão testadas, TRX apresentou o maior rendimento absoluto. Tiorredoxina é uma proteínas nativa de *E. coli* que, quando fundida a um peptídeo heterólogo aumenta a solubilidade citoplasmática da proteína de fusão, evitando que sejam precipitadas em corpos de inclusão. O aumento da solubilidade é necessário para a formação correta das ligações dissulfídicas nativas, também melhora significativamente o rendimento de proteína fundida (ELMORJANI et al., 2004). Combinando a tioredoxina a uma defensina de girassol (Ha-DEF1), Zélicout et ai. (2007) foram capazes de recuperar e peptídeos solúveis ativos a partir do lisado celular de *E. coli*.

Apesar de alguns bons resultados com outros AMPs (MOON; HENZLER-WILDMAN; RAMAMOORTHY, 2006; SRINIVASULU et al., 2008), fusões GST são altamente susceptíveis à degradação proteolítica resultando numa baixa produção de defensina em *E. coli* (CHEN et al., 2008). Tem sido relatado que o efeito das

proteínas carreadoras no rendimento final da expressão podem variar de acordo com a proteína fundida a ela (HAMMARSTRÖM et al., 2002).

Alguns sinais de secreção podem ser adicionados à defensina recombinante para dirigir a sua exportação para o periplasma de *E. coli*, ou mesmo dirigir a migração do peptídeo recombinante e sua atividade biológica para o citoplasma de uma célula alvo (CHEN et al., 2008; RODRÍGUEZ; ASENJO; ANDREWS, 2014), mas proteínas carredoras e sinais de secreção não são infalíveis. Kovalskaya e Hammod (KOVALSKAYA; HAMMOND, 2009) construíram cassetes utilizando o vector de expressão pET26b e dois AMPs de plantas, uma snakin (SN1) e uma defensina (PTH1) para a produção heteróloga em *E. coli*. O vector pET26b foi escolhido devido ao seu sinal *pelB* N-terminal, que deveria dirigir para uma localização periplasmática da proteína recombinante, para melhor “folding” e condições de solubilização. A estratégia falhou e toda a proteína recombinante foi encontrada concentrada em corpos de inclusão.

## 2.13 OS CORPOS DE INCLUSÃO (CIs)

Agregar defensinas recombinantes em corpos de inclusão pode ser uma outra estratégia para concentrar todos os peptídeos expressos e encurtar os passos de purificação. Aplicando a condição de lavagem apropriada, permite o isolamento de CIs formados por mais de 90% de proteína recombinante pura (SAMBROOK; RUSSEL, 2001). Alguns trabalhos propositadamente adicionaram carreadores indutores de agregação para conduzir à formação de CIs, alcançando um nível de proteína recombinante de 30% da proteína celular total (LEE et al., 2000). Kovalskaya e Hammond (2009) solubilizaram e realizaram o “refolding” de defensinas recombinantes armazenadas em CIs utilizando como agente redutor o DTT (ditiotreitol) e obtiveram moléculas ativa, capaz de inibir a o crescimento da bactéria *Clavibacter michiganensis* em contrações de 7 µM e do fungo *Colletotrichum coccoides* a 14 µM.

Alguns peptídeos podem ser fundidos à sua construção para aumentar a formação de CIs. Expressando um peptídeo fundido a uma cetoesteróide isomerase marcada com um hexa-histidina (para permitir sua remoção enzimática a posteriori) aumenta a insolubilidade da proteína recombinante, levando a sua alocação em Cis, que podem ser purificados posteriormente, alcançando uma elevada produtividade

de mais do que 30 mg de peptídeo purificado por grama de células (peso seco). Estes resultados são adequados para a produção de peptídeos para pesquisa científica, e atinge uma escala de pureza tal, que permite o uso dos mesmos em ensaios biológicos utilizados na investigação terapêutica (RODRÍGUEZ; ASENJO; ANDREWS, 2014).

# 3 **OBJETIVOS**

## 3.1 OBJETIVO GERAL

Produzir uma defensina recombinante (DeHys), utilizando um sistema de expressão heteróloga baseado em células de *E. coli*, a partir de uma sequência isolada de *Euphorbia hyssopifolia.*

## 3.2 OBJETIVOS ESPECÍFICOS

1) Otimizar a sequência de DNA do gene da defensina de *Euphorbia hyssopifolia* (DeHys) para expressão em *E. coli;*
2) Construir um vetor plasmidial de expressão em pET102/D-TOPO contendo o gene sintético para a produção da DeHys recombinante;
3) Produzir a proteína *DeHys* recombinante usando a linhagem BL21(DE3) de E. coli;
4) Purificar a proteína recombinante usando cromatografia de afinidade e isolar a porção da defensina DeHys dos demais elementos fusionados (tioredoxina e cauda de histidina).

# 4 CAPÍTULOS

## 4.1 CAPÍTULO I

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*(Aceito para publicação na Current Protein & Peptides Science)*

**Heterologous Expression Systems for Plant Defensin Expression: Examples of Success and Pitfalls**

**Heterologous Expression Systems for Plant Defensin Expression: Examples of Success and Pitfalls**

*Running Title:* Plant defensin heterologous expression

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**ABSTRACT**

Defensins are a superfamily of antimicrobial peptides, present in vertebrates, invertebrates, fungi and plants, suggesting that they appeared prior to the divergence in eukaryotes. The destitution of toxicity to mammalian cells of plant defensins has led to a new research ground, i.e., their potential medical use against human infectious diseases. Isolating defensins from natural sources, like plant tissues, can be time-consuming, labor intensive and usually present low yields. Strategies for large-scale production of purified active defensins have been employed using heterologous expression systems (HES) for defensin production, usually based in *E. coli* system. Like any other technology, HES present limitations and drawbacks demanding a careful experimental design prior the system selection. This review is proposed to discuss some of the major concerns when choosing to heterologously express plant defensins, with special attention on bacterial expression system.

**Key Words**: *E. coli, fusion*, carrier protein, secretion signal, inclusion bodies, antimicrobial peptides.

## INTRODUCTION

Organisms throughout all kingdoms (including prokaryotes, lower and higher eukaryotes) present an ancient defense mechanism based on the secretion of small antimicrobial proteins. These proteins are called Antimicrobial Peptides (AMPs) and act inhibiting the growth of bacteria, fungi, parasites and viruses. Together with other defense mechanism, they form the innate immune system, protecting the host against microbial attacks [1].

AMPs are gene-encoded and they are either constitutively expressed or rapidly transcribed upon induction by the presence of invading microbes or their products. AMPs are classified according their function and structure, which are determined by the amino acids residues on the primary sequence of the protein (glycine, cysteine, histidine, proline, tyrosine, arginine, lysine and serine) [2]. Defensins are a superfamily of the AMP, and can be found in vertebrates, invertebrates, plant and fungi suggesting that it appeared prior to the divergence in eukaryotes [3]. Although defensins present a low sequence similarity, they share highly conserved α/γ c-core primary sequence motifs [4] and a cysteine-stabilized α/β (CSα/β) tertiary structure [5]. There are eight typical cysteine residues that form four disulfide bonds providing stability to the defensin [6].

Isolating defensins from natural sources, like plant tissue can be time-consuming, labor intensive and usually do not result in high yields [7]. Therefore, other strategies for large-scale production of satisfactory amounts of purified active defensins have been employed, such as the use of recombinant DNA technology [8], which has become a promising and rapidly expanding area. A growing number of studies have reported the use of different heterologous expression systems (HES) for defensin production. However, like any other technology, HES has also been target of practical limitations and drawbacks (in both plant bacteria or fungi platforms) demanding a careful experimental design prior the system selection. In this context, the present review article aims to discuss the important elements involved in the production of recombinant plant defensins with a special focus on the *Escherichia coli* heterologous expression system. The presented data include steps for obtaining the peptide coding sequence until its isolation and purification.

## 1. PLANT DEFENSINS

The first plant defensins were discovered in 1990, in wheat and barley seeds [9], initially termed δ-thionin due to their similar size and number of disulfide bonds to α-thionins and β-thionins. Mostly, defensins present three to five disulfide bonds, which stabilize an antiparallel beta-sheet flanked by an alpha-helix thus conferring resistance against extreme pH and temperatures [10].

Defensins are small (<10 kDa, 45-54 amino acids), often highly basic, cationic, cysteine-rich peptides and participate of the innate immune system of plants, as an ancient strategy of protein secretion for protection against invading microorganisms [11]. Defensins exhibit a variety of biological activities, especially antifungal and antibacterial activity [12] requiring a low minimal inhibitory concentration [13]. Seeds are energetic substances storage organs, becoming target to a wide variety of heterotrophic organisms, especially fungi. Therefore, defense mechanisms based on cysteine-rich antimicrobial peptides are highly represented in seeds. Recent studies have found new *in vivo* roles for plant defensins: protective role in seeds germination by creating a microenvironment around the seed in which fungal growth is suppressed [14]; protective role in embryo

formation by local expression in the embryo-surrounding right after pollination [15]; protective role against biotic stress, including fungi [16], bacteria [17], herbivore insects [18], nematodes [19] and parasitic plants [20] as well as role in symbiotic interactions (e.g. down-regulation in root when in the presence of nitrogen fixing bacteria [21]). A recent study using *Prunus persica* (peach) defensin, PpDFN1, found that the addition of a neutral sphingolipid ceramide β-D-galactoside to a lipid monolayer of egg-phosphatidylcholine (ePC) increased significantly the defensin affinity to the artificial membrane. Nevertheless, when the same experiment was made using lipids isolated from phytopathogenic fungi, the affinity presented was even higher, demonstrating that the bounding of a plant defensin to target pathogen membrane is dependent of its lipid concentration and composition [5]. The same study showed no PpDFN1 defensin activity against human erythrocytes, in accordance with Wong and Ng [22] findings on vulgarin (a defensin isolated from *Phaseolus vulgaris*), which is known to devoid toxicity to mammalian spleen cells and erythrocytes. Also, in Gonçalves et al. [23] it is shown that *Pisum sativum* defensin, Psd1, presents no affinity to cholesterol-enriched lipid bilayers, just like the membranes found in mammalian cells. Some recent discoveries on plant defensin antifungal activity shows that such activity might be mediated by electrostatic interaction with anionic lipid components of fungal membranes [24].

Since their discovery, studies on defensins have been conducted regarding (i) activation pathway [25, 26], (ii) mode of action [13], and (iii) potential for improving plant resistance against phytopathogens [27]. Therefore, the knowledge about plant defensins destitution of toxicity to mammalian cells has led to a new research ground, that is, its potential medical use against human infectious diseases [22, 28, 29].

### 1.1. Plant defensin prospection

Obtaining a mature plant defensin peptide sequence can be basically achieved by two main strategies: reverse genetics or database prospection. The reverse genetics strategy consists basically in the extraction and purification of native plant defensins from tissues followed by amino acid sequencing. Defensin sequence prospection (data mining) is based on the use of bioinformatic tools to find sequences with similar defensin motifs in genomic plant databases. In both cases, the knowledge of the defensin sequence will provide the information needed to design primer for cDNA amplification and/or artificial plasmids baring the mature defensin sequence.

#### 1.1.1. Reverse genetics strategy

The conserved structure and biochemical features of plant defensins allow the purification of previously unknown peptides from plants for which the genome sequences are still not available. Although seeds are the most commonly used tissue for defensin isolation [30, 31, 32], defensins have also being isolated from other plant organs. Lay et al. [33] isolated one defensin from ornamental tobacco and two from *Petunia*, in both cases using floral tissue. Through immunoblot analysis, they discovered that defensins were more concentrated at the ovaries, petals and pistils during the early stages of flower development. Defensins isolation was also reported on *Capsicum annuum* mesocarp of ripe fruits [17], on *Ipomoea batatas* (L.) Lam. 'Tainong 57' storage roots [34], on injured *Arabidopsis thaliana* [25] and also on intact *Spinacia oleracea* [35] leaves.

The process of extraction often consists in powdering the tissue, producing seed flour or macerating other tissues with liquid nitrogen, followed by the addition of a saline extraction buffer at low temperature (4°C). The

157 supernatant is then collected and exposed to different concentrations of ammonium sulfate [36] causing the
158 precipitation of groups of proteins based on their charges. The pellets precipitated at each ammonium sulfate
159 concentration must be resuspended and submitted to chromatographic methods in order to obtain a purified
160 sample of the target peptide [31]. Due to its cationic profile the sample passes through an affinity
161 chromatography column (weak anion exchange column) and the fraction containing the defensins is then applied
162 to a size-exclusion chromatography column (a process also referred as gel-filtration chromatography) [37].
163
164 **1.1.2. Databases prospection strategy**
165 The increasing drug-resistance of microbial pathogens has created a necessity for the discovery of new
166 antimicrobial drugs. Antimicrobial peptides, especially plant defensins, have been proved to be effective against
167 many bacterial and fungal pathogens and to present no toxicity against mammalian cells. Therefore, the
168 development of AMPs and defensins databases has helped many researchers on their studies, providing not only
169 sequences, but tools to enhance the way the information is presented leading to a better understanding of their
170 protein of interest. Some databases bare programs to perform homology search, multiple sequence alignment,
171 phylogenies, physicochemical profiles, and other calculations and predictions [38].
172 An example of database is the PhyAMP - a plant specific data repository for natural plant antimicrobial
173 peptides. Records for 55 plant defensins can be found in their database, along with very useful diagrams and
174 charts concerning defensins phylogenetic trees, base and acid amino acid distribution and activity. Tools to make
175 the prospection easier or to provide further data for study, as similarity search, hidden Markov Models,
176 physicochemical profile and sequence alignment are available at PhyAMP [39]. In some cases, if a researcher is
177 acquainted to the protein he is looking for, some non-specific databases such as GenBank
178 (www.ncbi.nlm.nih.gov/genbank), can be also helpful [40].
179 As an example, Karri and Bharadwaja [40] searched GenBank to find the sequences of Tfgd2 (*Trigonella*
180 *foenum - graecum* defensin 2; GenBank accession number AY227192) and RsAFP2 (*Raphanus sativus*
181 antifungal protein 2; GenBank accession number U18556) to construct the fusion gene Tfgd2-RsAFP2 (that was
182 later deposited at GenBank under the accession number KF498667). The constructed Tfgd2-RaAFP2 proved to
183 be three times more efficient against *Phaeoisariopsis personata* conidia germination. A list presenting some of
184 the available AMPs and defensins databases is provided on Table **1**.

## 2. HETEROLOGOUS SYSTEMS FOR PLANT DEFENSIN PRODUCTION

Advances in recombinant DNA technology have provided an opportunity to produce high levels of plant defensins. This technology enables cloning of foreign genes in specific vectors for expression in prokaryotic and/or eukaryotic systems, and considered the most effective method regarding time and production costs [41]. Proteins have several features that should be carefully observed when choosing a host system for heterologous production, such as size, intracellular localization or secretion, proper folding, and glycosylation pattern [42].

The main hosts used for AMPs production are bacteria and yeasts, representing 97.4% of heterologous-expressed AMPs [42, 43]. More recently, plants have emerged as a promising host for AMPs production since transgenic plants can be directly used for microbial control expressing the peptide in the desired crop [42, 44].

Different hosts have been used for the production of plant defensins in prokaryotic and eukaryotic systems. Plant defensins have already been produced in bacteria [5, 45, 46], yeasts [47-49] and transgenic plants [50-52] with variable degrees of success and protein yield.

### 2.1. *Escherichia coli* expression system

*Escherichia coli* is the most employed microorganism for heterologous protein production. This system is the first choice as host for plant defensin production and so far the one with highest yields reported. There are many reasons for that: *E. coli* has proven to be the most cost-effective method of recombinant protein production due to its rapid growth, large availability of commercial expression vectors, well-established DNA manipulation protocols and extensive knowledge regarding its genetics, biochemistry and physiology [53]. However, there are some pitfalls that need to be overcome in order to achieve effective production in bacteria.

Plant defensins have already been produced heterologously in *E. coli* expression systems. Nanni et al. [5] achieved a yield of 0.5 mg.L-1 of the cloned PpDFN1 using the pET-32 vector (Novagen) and *E. coli* BL21 (DE3) Origami pLys cells (Novagen). The produced defensin was functional and displayed antifungal activity against *Botrytis cinerea*, *Monilinia laxa* and *Penicillium expansum*, with IC50 values of 15.1, 9.9 and 1.1 $\mu$ g.mL-1, respectively.

Similarly Kovaleva et al. [54] had already expressed the *Pinus silvestris* defensin 1 (*Ps*Def1) in the pET system (pET42a) and BL21 (DE3) *E. coli* strains, although with a fusion protein glutathione-S-transferase (GST). They were able to produce a full length defensin which was not biologically active when fused to GST but strongly functional against a panel of pathogenic fungi when separated from the fusion protein.

In the following sections, it will be discussed in detail some approaches that have to be considered for *E. coli* heterologous expression.

#### 2.1.1. *Escherichia coli* Strains

The lack of comparative studies of recombinant plant defensin expressed in different strains makes difficult to predict which strain will provide better results. Choosing the right expression system may be crucial to achieve a satisfactory yield of a recombinant defensin. Sometimes, a preliminary assay using different strains can help making such decision, as recombinant protein yield can vary from a discreet to a discrepant 10-fold difference among strains [55].

Defensins present eight cysteine residues that interact creating four disulfide bonds, therefore, it is important that the chosen expression strain is able to form such fold. The disulfide bond formation is inhibited in the *E. coli* cytoplasm, because of the presence of thioredoxins, and it is dependent of an enzyme alkaline phosphatase, which is only active in the periplasm [56]. Cytoplasmic expression and correct folding of defensins in *E. coli* has become possible through the use of thioredoxin reductase (trxB) mutant strains and even more if periplasmic isomerases, such as the enzyme disulfide-bond isomerase (DsbC, catalyze the formation and correction of disulfide bonds; also called foldases) were engineered to be expressed in the cytoplasm as well [57]. Some strains present plasmids encoding for rare tRNAs attenuating the codon bias, making codon optimization less crucial for a successful expression [58]; others provide a more oxidized environment in the cytoplasm enhancing the recombinant defensin disulfide bond formation due to mutations in the trxB and glutathione reductase (gor) genes [59]. There are also strains available in the market that combine these two modifications, for a better expression control and cytoplasmic disulfide binding formation of heterologous proteins expressed in *E. coli* [60].

Protein degradation during purification steps can be minimized when using strains mutated for the Ion protease and the ompT outer membrane protease [55]. It is important to highlight that even with optimal experimental design it is not possible to predict the outcome of a successful soluble recombinant defensin production. Kovalskaya and Hammod [61] constructed cassettes using the pET26b expression vector and two plant AMP's, a snakin (SN1) and a defensin (PTH1), for heterologous production in BL21 (DE3) strains. The strategy to produce soluble molecules failed and the recombinant proteins were found to be concentrated in the Inclusion Bodies (IBs).

There is a myriad of expression strains available, each one with different combination of strategic modifications: RNAse E mutated strains for a longer intracellular RNA lifespan, T7 Lysozyme for better control of toxic peptides, salt-induced promoters for better solubility, lacY mutants for homogeneous induction levels in each cell and accurate expression control and addition of DsbC chaperone for better cytoplasmic folding (Table **2**).

**2.1.2. Vectors**

It is important to highlight that one must try to combine strains and expression vectors strategies to make the expression more prone to success. For example, the use of expression vectors baring a N-terminal *pelB* signal is recommended when working with lon protease and the ompT outer membrane protease mutated strains, providing a periplasmic location for the recombinant protein, for better folding and solubilizing conditions [62].

Expression vectors can add binding molecules to the recombinant protein making protein purification less time consuming and laboring. The pMAL expression vectors (commercialized by New England BioLabs), for instance, are divided in two major groups: the pMAL-c and the pMAL-p vectors. Both groups add a maltose binding protein to the recombinant defensin in a single-step purification process. The pMAL-c vectors (as the pMAL-c2x, pMAL-c5x, etc.) present the deletion of the *malE* signal sequence resulting in cytoplasmic expression of the fusion protein. It leads to better yields (recombinant protein levels of 20-40% of total cell protein), but it is not recommended for peptides that require disulfide bond formation [17]. The pMAL-p series bares the intact signal sequence of the *malE* gene, leading to the secretion of the recombinant peptide to the periplasm. The pMAL-p series shows lower yields (recombinant protein levels of 1-20% of total cell protein),

but is more prone to form the correct disulfide bond folding.

For plant defensin heterologous expression, the most widely used expression system is the pET system, initially developed by W. F. Studier and B. A. Moffatt in 1968, that created an RNA polymerase expression system which was highly selective for bacteriophage T7 RNA polymerase [63]. These vectors have as main feature a strong bacteriophage transcription signal, leading to a high level of heterologous expression, with the recombinant protein comprising up to 50% of total cell proteins a few hours after induction. These vectors must be cloned into strains that have the T7 Polymerase gene for expression. Strains, like BL21 (DE3) were engineered to present the T7 polymerase gene under the control of a *lac* promoter derivate L8-UV5 lac [63]. The derivate L8-UV5 *lac* promoter contains three point mutations, which increase promoter strength, decrease its dependence on cyclic AMP and creates a stronger promoter that is less sensitive to glucose. These modifications permit a strong induction of T7 RNA polymerase using IPTG as inducer [65].

### 2.1.3. Carrier proteins and secretion signals

It is not surprising that expressing an antimicrobial peptide in a prokaryotic system can lead to the death of the hosts strains themselves. The use of a carrier protein helps minimizing the toxic effects of the expressed defensin in the host cells. Because of its reduced size, defensins are prone to be degraded by intracellular proteases. Such degradation can be overcome by fusioning a carrier protein to the heterologous peptide. The carrier protein mimics the *E. coli* native proteins pro-segments protecting the fused protein from intracellular proteases attack.

The two most often used carriers for soluble expression in *E. coli* are the thioredoxin (TRX) and the glutathione-S-transferase (GST). Bogomolovas et al. [66] demonstrated that among 13 tested fusion partners, TRX presented the highest absolute yield. Thioredoxin is a resident *E. coli* protein that when fused to a heterologous peptide enhances the cytoplasmic solubility of the fusion protein keeping it from precipitating as inclusion bodies. The increase of the solubility is required for the proper formation of native disulfide bonds, also improving significantly the fused protein yield [67]. Fusing the thioredoxin to a sunflower defensin (Ha-DEF1), Zélicout et al. [20] were able to recover soluble and active peptides from *E. coli* cell lysate.

Despite many successful results with other antimicrobial peptides [68, 69], GST fusions are highly susceptible to proteolytic degradation resulting in inefficient protein production in *E. coli* [70]. To date, no carrier protein has been directly correlated with a higher yield of recombinant peptides. It has been reported that the effect of carrier proteins may vary according to the specific protein fused to it [71].

Some secretion signals can be added to the recombinant defensin to direct its allocation in the *E. coli* periplasm, or even direct the recombinant peptide migration and biological activity to the cytoplasm of a target cell [70, 72], but carrier proteins and secretion signal are not infallible. Kovalskaya and Hammod [61] constructed cassettes using the pET26b expression vector and two plant AMPs, a snakin (SN1) and a defensin (PTH1) for heterologous production in *E. coli*. The pET26b vector was chosen due to its N-terminal *pelB* signal, which was supposed to provide a periplasmic location for the recombinant protein, for better folding and solubilizing condition. The strategy failed and all the recombinant protein was found to be concentrated in the IBs.

### 2.1.4. Inclusion bodies

The recombinant defensin aggregated in inclusion bodies can be another strategy to concentrate all the expressed peptides and shorten the purification steps. Applying the appropriate washing condition allows the isolation of the IBs containing more than 90% pure recombinant protein [73]. Some works purposely add aggregation-inducer carriers to drive the formation of IBs, achieving a recombinant protein level of 30% of the total cell protein [74]. Kovalskaya and Hammond [61] solubilized and refolded the recombinant defensin stored in IBs using the reducing agent DTT (Dithiothreitol) and obtained an active molecule, able to inhibit bacterial (*Clavibacter michiganensis*) at 7 μM and fungal (*Colletotrichum coccoides*) growth at 14 μM concentration.

Some peptides can be fused to your construction to enhance IBs formation. Expressing a peptide fused to a hexahistidine tagged ketosteroid isomerase (to be removed enzymatically later) increases the insolubility of the produced peptide, leading its allocation in IBs, which can be purified later, achieving a high productivity of more than 30 mg of purified peptide per gram of dry cell weight. These results are suitable for the production of peptides for scientific research, as it achieves the required scale and purity, even for biological assays used in therapeutic peptides research [72].

## CONCLUDING REMARKS

All efforts to develop novel or improved techniques that successfully achieve high levels of protein strongly favor bacteria as a host for the large scale production. However, it is known that if the peptide to be expressed requires certain types of post-translational modifications, which are not performed in prokaryotes, different hosts for defensin production have to be considered. Moreover, there are some challenges that need to be overcome in *E. coli* recombinant protein production, such as protein insolubility and purification steps.

In some cases, yeasts such as *Pichia pastoris* showed some advantages over *E. coli*, particularly in regard to post-translational modifications (specially, glycosylation), increasing the solubility and producing large quantities of heterologous protein. In addition, *P. pastoris* allows secretion of the recombinant protein, which results in less purification steps; did not require a carrier protein as reported for *E. coli*, which may imply facilitated scale-up processes and, also, can be cultivated at a high cell density with no toxic products or ethanol production using cost-effective culture media. Table 3 shows some important points on plant defensin heterologous expression in *Pichia pastoris* and *Escherichia coli*.

Nevertheless, there is a limited variety of plant AMPs produced in *P. pastoris* with the amount of recombinant peptide produced varying from 55 μg to 748 mg.L−1 of culture media [75]. Thus, *P. pastoris* is definitely a good choice for host expression, when the *E. coli* expression system was not satisfactory, although it still requires the establishment of a variety of promoters and strains in association with process development in order to achieve higher yield of protein.

## CONTRIBUTION OF AUTHORS

LRSG, did the literature search and wrote the manuscript. VP and ALSJ, helped in the literature search and reviewed the draft manuscript. SC and AMBI edited the manuscript and assisted as consultants on defensins and plant defensins, respectively. ACF, mastermind of the manuscript, structured the way the review, revised the draft and final manuscript. All authors approved the final version of the manuscript.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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**LIST OF ABBREVIATIONS**

HES: heterologous expression systems; AMPs: Antimicrobial Peptides; PpDFN1: Prunus persica defensin; ePC: egg-phosphatidylcholine; Psd1: Pisum sativum defensin; Tfgd2: *Trigonella foenum-graecum* defensin 2; RsAFP2: *Raphanus sativus* antifungal protein 2; DsbC: disulfide-bond isomerase; GST: protein glutathione-S-transferase; trxB: thioredoxin reductase; gor: glutathione reductase; IBs: inclusion bodies; TRX: thioredoxin; Ha-DEF1: sunflower defensin; DTT: Dithiothreitol.

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Table **1.** Antimicrobial peptides and defensins databases.

| Database | Year* | Summary | URL |
|---|---|---|---|
| **LAMP** | 2013 | LAMP: a Database Linking Antimicrobial Peptides. | http://biotechlab.fudan.edu.cn/database/lamp |
| **DAMPD** | 2011 | DAMPD: an update and a replacement of the ANTIMIC database. | http://apps.sanbi.ac.za/dampd |
| **CAMP** | 2009 | Collection of Antimicrobial Peptides, India. | http://www.bicnirrh.res.in/antimicrobial |
| **RAPD** | 2008/09 | A database of recombinantly-produced antimicrobial peptides, USA. | http://faculty.ist.unomaha.edu/chen/rapd/ |
| **APD2** | 2004/09 | The Antimicrobial Peptide Database, USA | http://aps.unmc.edu/AP/main.html |
| **PhyAMP** | 2008 | A database dedicated to plant antimicrobial peptides, Tunisia. | http://phytamp.pfba-lab-tun.org/ |
| **Defensins** | 2007 | Defensins Knowledgebase, Singapore. | http://defensins.bii.a-star.edu.sg/ |
| **AMPer** | 2007 | A database and discovery tool for antimicrobial peptides, based on hidden Markov models and the SwissProt databank, Canada. | http://marray.cmdr.ubc.ca/cgi-bin/amp.pl |
| **AMSDb** | 2002/04 | Antimicrobial sequence Database, Italy. | http://www.bbcm.univ.trieste.it/~tossi/amsdb.html |

* Creation and/or update of the database

622 Table **2.** *Escherichia coli* strains used in plant defensin heterologous expression.

| **Bacterial Strain** | **Features** | **Manufacturer** | **Reference** |
|---|---|---|---|
| **BL21 (DE3)** | High Transformation efficiency, IPTG induction, deficient of Lon and OmpT proteases. Suitable for non-toxic products expression. | Novagen/ Stratagene | [69, 70] |
| **BL21 (DE3)-pLysS** | Same as BL21 (DE3) with the addition of a plasmid, pLysS, which lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following IPTG induction. It is therefore suitable for expression of toxic genes. | Novagen/ Stratagene | [35,58,73,74] |
| **Origami 2** | Origami™ 2 host strains are K-12 derivatives that have mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, which greatly enhance disulfide bond formation in the *E. coli* cytoplasm and are recommended only for the expression of proteins that require disulfide bond formation for proper folding. | Novagen | [8,19,75] |
| **Origami B** | Origami B host strains carry the same trxB/gor mutations as the original Origami strains, except that they are derived from a lacZY mutant of BL21. | Novagen | [76] |
| **Origami B (DE3)pLysS** | Same as Origami B with the addition of a plasmid, pLysS, which lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. Thus it is suitable for expression of toxic genes. | Novagen | [5,77] |
| **Rosetta (DE3)** | Rosetta host strains are BL21 lacZY (Tuner) derivatives designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. These strains supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA on a compatible chloramphenicol resistant plasmid. The tRNA genes are driven by their native promoters. | Novagen | [51,78] |
| **Rosetta (DE3)pLysS** | In Rosetta (DE3)-pLysS, the rare tRNA genes are presented on the same plasmids that carry the T7 lysozyme. | Novagen | [55] |
| **Rosetta-gami(DE3)LysS** | Rosetta-gami host strains are Origami derivatives that combine the enhanced disulfide bond formation resulting from trxB/gor mutations with enhanced expression of eukaryotic proteins that contain codons rarely used in *E. coli.* These strains supply tRNAs for AGG, AGA, AUA, CUA, CCC, GGA on a compatible chloramphenicol-resistant plasmid. The tRNA genes are driven by their native promoters. In Rosetta-gami(DE3)-pLysS, the rare tRNA genes are presented on the same plasmids that carry the T7 lysozyme. | Novagen | [55,79] |
| **BL21** | BL21-CodonPlus-RIL chemically competent cells carry | | [8,80,81 |

| | | | |
|---|---|---|---|
| **CodonPlus** | extra copies of the argU, ileY, and leuW tRNA genes. The tRNAs encoded by these genes recognize the AGA/AGG (arginine), AUA (isoleucine), and CUA (leucine) codons, respectively. | Stratagene | ] |
| **AD494** | AD494 strains are thioredoxin reductase (trxB) mutants of the K12 strain that enable disulfide bond formation in the cytoplasm, providing the potential to produce properly folded active proteins. | Novagen | [82] |
| **BL21trxB** | BL21trxB strains possess the same thioredoxin reductase mutation (trxB) as the AD494 strains in the protease deficient BL21 background. The trxB mutation enables cytoplasmic disulfide bond formation. | Novagen | [8] |

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624 Table **3.** Heterologous expression in *Pichia pastoris* Vs. *Escherichia coli*.

| **Organism** | ***Pichia pastoris*** | ***Escherichia coli*** |
|---|---|---|
| **Transcription regulation** | Using the AOX promoter, the transcription of the foreign gene can be tightly regulated. | Most of the vectors present a detectable basal transcription of the recombinant gene. |
| **Protein purification** | Proteins can be easily secreted in the culture medium, there for; it can be purified in a single step. | Proteins usually need to be purified from cell lysate or inclusion bodies, which can be hard laboring. |
| **Protein folding** | Eukaryotic proteins with disulfide bonds can be correctly formed with no extra treatment. | A refolding procedure may be necessary, especially when the expressed protein is in the IBs. |
| **Strains** | *Pichia* still present a limited variety of expression strains. | There is a great list of engineered E. coli strains to choose accordingly to your goal. |
| **Expression vectors** | There is a single commonly used expression vector (integrative plasmid). | Many options of expression vectors available. |

## 4.2 CAPÍTULO II

*(Artigo para submissão na Current Protein & Peptides Science)*

# **Identification, Isolation, Cloning and Expression of a Defensin Gene (DeHys) from *Euphorbia hyssopifolia* in *E.coli***

# **Identification, Isolation, Cloning and Expression of a Defensin Gene (DeHys) from *Euphorbia hyssopifolia* in *E.coli***

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**ABSTRACT**

Plants have complex and efficient defense mechanisms against many pathogens, among them, the transcriptional activation of numerous genes, including defensins. Defensins are small (ca. 5 kDa), basic and cysteine rich peptides, participating in the plant defense system. The potential of using plant defensins as new therapeutic substances comes from its structural similarity with mammalian defensins; broad antimicrobial spectrum; fast activity in low doses; synergism with other defensins and therapeutic schemes. *Euphorbia hyssopifolia* L. is a weed widely used in Brazilian, Indian and Asian tradicional medicine, standing out by its antimicrobial activity.

However, the obtention of plant defensins from natural sources, particularly for medicinal or biotech purposes, is an inappropriate process due to their low abundance and the presence of other compounds that can be toxic, leading to the need of extra caution on its use. Thus chemical synthesis and/or heterologous expression systems (including *E. coli* and *P. pastoris*) have been the techniques widely used to obtain large amounts of functionally active peptides, especially for therapeutic application. In this work we identified, isolated, cloned, and heterologously expressed a defensin from *E. hyssopifolia* (DeHys). The sequence retrieved presented 361 bp disposed in one intron and two exons. A propeptide with 78 amino acids was generated from the coding sequence, which presented 237 bp. The mature peptide had 47 amino acids, and obeyed the alpha-beta cysteine stabilized (CSαβ) motif previously described for defensins. The nucleotide and amino acid sequences from E. hyssopifolia presented high homology with defensin sequences available at databases. The 3D analysis revealed the characteristic disulfide bonds, hydrophobicity, electrostatic potential and solvent accessibility. At the end of the present study a recombinant 24 kDa protein containing the mature peptide of E. hyssopifolia defensin was heterologously expressed. Therefore, this work contributed to the knowledge of a novel defensin that might help the development of new pharmaceutical products.

**Key Words:** *Antimicrobial peptide, medicinal proteins, plant genetic, E. coli, expression, defensin.*

## 1. INTRODUCTION

Superior plants has complex and efficient defense mechanisms against several pathogens, which leads to transcriptional activation of a cascade of genes in different pathways, including the ones involved in defensins production [1]. They are small proteins (ca. 5 kDa), basics and cystein rich that make part of the defense arsenal of these organisms. Some defensins are constitutively expressed, while others are developmentally regulated or induced by different factors, including abiotic agents as cold, drought, heavy metals, potassium starvation, among others - as well as the presence of microbial pathogens [2].

The diversity and disseminated occurrence of defensins in vegetal kingdom indicate that they are a rich font of proteins with biological activities potentially important to both, agrobiotechnological and pharmacological applications. According to Carvalho & Gomes [3], among these characteristics are antimicrobial, insecticidal and antiparasitary activities, making these peptides good candidates for proteomic engineering and production of transgenic plants, which may withstand pathogens and pests, while the codified proteins are non-toxics to mammals, or plants. Furthermore, these proteins are active against many pathogens and can work synergistically with others antimicrobial compounds.

Specimens of Euphorbia hyssopifolia L. are found virtually all over the Brazilian territory [4], being considered a weed, with recognized medicinal potential [5, 6, 7] and some toxicity [8]. This plant is used in Indian [9], Asiatic [7] and Latin American [6] folk medicine and is noted for their antimicrobial activity. In Brasil, it is used as diuretic, to treat urinary infections and to remove warts as well [5, 10]. As noticed by Jha et al. [11], the studies on isolation and characterization of defensins refer in most cases to crop species. *E. hyssopifolia* happens to be one of the first wild species to undergo this type of study. Besides its well documented therapeutic potential, it is itself resistant to a broad spectrum of environmental and biotic stresses, thus revealing its potential as a source of genes of interest for the development of a new variety of transgenic plants, also allowing the conception of new medicines for several human diseases. However, the toxicity of other *E. hyssopifolia* compounds leads to the need of extra caution on its use [8], being the production of purified defensin with antimicrobial activity, a safer application of its therapeutic properties.

The present work aimed to identify, isolate, clone and expres a defensin from *E. hyssopifolia* (DeHys) using a heterologous expression system based on *E. coli* BL21(DE3) cells.

## 2. MATERIAL AND METHODS

### 2.1. *DNA Extraction from E. hyssopifolia*

Genomic DNA was extracted from young leaves of E. hyssopifolia as described by Weising et al. [12], followed by precipitation of polisaccharydes (10 mM Tris-HCl, pH 8,0; 25 mM NaCl,) as described in Michaels et al. (1994) and, finally treated with RNAse A (10 µg/mL). Quantity and integrity of the isolated DNA were evaluated by spectrophotometer and electrophoresis in agarose gel 1.2 %, respectively.

### 2.2. *PCR amplifications, cloning and sequencing*

The amplification of a gene of a defensin from E. hyssopifolia (DeHys) was achieved using the forward primer 5'-TCCATGGCTCGCTCTGTGTCTT-3 and reverse primer 5'-TGAAGTTTTAACAGTGTTTGGTGCACAAG-3', designed by Padovan et al. [13] based on the ESTs (Expressed Sequence Tags) sequence from Phaseolus vulgaris (TC147/TiGER) and employed by these authors for heterologous PCR in *V. unguiculata*. Amplification reactions followed the described by the authors but reducing the annealing temperature to 55°C to decrease specificity. The fragment was visualized on 1.2 % agarose gel and quantified using Lambda/Hind III (λ DNA) DNA as reference.

The fragments were inserted into the pGEM -T Easy Vector System I (Promega, Cat A1360) as recommended by the manufacturer. Ligation products were transformed into Max Efficiency® DH10B™ Competent Cells (Invitrogen, Carlsbad, CA, USA) according to the manufacture's recommendation. After transformation the bacterial cells were plated on LB agar plates containing 100 mg L-1 ampicillin and incubated at 37ºC overnight. Colonies containing recombinant plasmids were identified by PCR using SP6 and T7 primers, which flank the insert in plasmid pGEM-T. PCR conditions were performed using an initial denaturation at 94ºC for 2 min, followed by 35 cycles of: denaturation (15 s at 94°C), primer annealing (20 s at 55°C) and extension (30 s at 72°C), followed by a final extension at 72°C for 5 min, in a Techne TC-412 termocycler (Barloworld Scientific). The PCR products were visualized on 2% agarose gel, using the 50 bp DNA Ladder (Promega). Plasmid DNA from positive clones was isolated from overnight grown cultures using an alkaline lysis method [14].

Sequencing was carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in the automated sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA-USA). The sequencing reactions were conducted using vector-based primers (T7 and SP6) in both directions

### 2.3. *Bioinformatics Sequences Analysis*

The multiple sequence alignment of nucleotides sequences of each clone was carried out using the program ClustalW [15]. These alignments were screened to generate consensus sequences to obtain the maximum length in base pairs with quality. The trimmed nucleotide sequence had the gene structure analyzed and translated into amino acids by the program fGenesh (HMM-based gene structure prediction) (http://linux1.softberry.com/berry.phtml).

#### 2.3.1. *Sequences of coding gene and putative defensin*

The nucleotide sequence encoding the defensin of *E. hyssopifoli*a was aligned with the tools BlastN and BlastX against sequences available in GenBank (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the genome of the species from core Fabidae available in Phytozome v7.0 (http:// www.phytozome.net/). From these, the ESTs and genomic sequences most similar were compared to sequences of DNA from *E. hyssopifolia*. Proteins encoded by those genes and their similarity were analyzed.

#### 2.3.2. *Defensin properties analysis*

To predict the cleavage site for signal peptide the program Philius Transmembrane Prediction Server (http://www.yeastrc.org/philius/pages/philius/ runPhilius.jsp) was applied [16]. The disulfide bridges were predicted with DISULFIND program [17] and multiple alignment of protein sequences was made in the ClustalX2 program [14] and ESPript 2.2 [18], being the former used to distinguish the hydrophobic and charged amino acids, while the last aimed the prediction of secondary structure of aligned proteins. Comparative analyzes of the proteins properties, with and without the signal peptide were performed using the programs APD2: Antimicrobial Peptide Predictor [19] and ProtParam [20]. The estimated molecule charge in different pHs was predicted by the Protein Calculator v3.3 tool (http://www.scripps.edu/~cdputnam/protcalc.html). Information about the electrostatic potential and accessibility of the molecule were obtained through the program DeepView v4.04 [21], employing the Poisson-Boltzmannn method to calculate the electrostatic potential, placing 4 as a dielectric constant of protein and 80 to the solvent (water). At Jmol Viewer 12.0 (http://jmol.sourceforge.net/) molecule was edited to display the differential structure of the molecule and coloring of the amino acid residues.

#### 2.3.3. *Modelling*

A BlastP from the mature peptide sequence was performed in PDB database (RCSB Protein Databank) (http://www.rcsb.org/pdb/home/home.do) in order to choose the structures with the most sequence similarity to serve as models on the 3D-modeling of the protein. The three-dimensional structure of the defensin was modeled using the Modeller v 9.7 program [22], while the relaxation of the structure occurred by molecular dynamics, using the program VMD 1.9 [23]. The quality evaluation of the model obtained was performed with the tool Protein Structure & Model Assessment from SWISS-MODEL Workspace (http://swissmodel.expasy.org/) [24] from data of QMEAN6 global score [25] and Z-score [26]. While using PROCHECK v.3.5.4 [27] were obtained the Ramachandran plot of the structure and its G-factor [28].

### *2.4. Heterologous Expression*

#### 2.4.1. *Synthetic gene, PCR and expression vector construction*

A plasmid (pIDTSmart-Amp) harboring the synthetic *E. hyssopifolia* DeHys was ordered from IDT (Integrated DNA Technology). The synthetic gene consisted of the defensin mature peptide sequence (no introns) with optimized codons for *E. coli* expression. Specific primers were designed to amplify the synthetic gene from the pIDTSmart Amp: forward primer 5'-**CACC**ATGAGGACATGTGAGTCTCAGAGCCACCGTTTC-3' and reverse primer 5'-CCTTCCCTCGATACAGTGTTTGGTGCACAAGCA-3'. The first four nucleotides (CACC overhang) in the

forward primer were necessary for cloning using the TOPO approach used in the pET102/D-TOPO expression vector, while 12 underlined nucleotides in the reverse primer code for a factor Xa protease site.

PCR amplification was performed using 1X Phusion High-Fidelity DNA Polymerase Buffer, 200 μM dNTPs, 0.5 μM each primer, 10 ng of pIDTSmartAmp + DeHys and 1U of Phusion HighFidelity DNA Polymerase (Thermo Scientific) in a final volume of 50 μl. The PCR conditions were as follows: 98$^{o}$C for 30 s (denaturation), 25 cycles of 98$^{o}$C for 10 s, 62$^{o}$C for 30 s and 72$^{o}$C for 30 s; followed by a final extension at 72$^{o}$C for 5 min

#### 2.4.2. *Subcloning and Construction Confirmation*

The PCR product was purified using Wizard® SV Gel and PCR Clean-Up System (Promega) as described by the manufactor and subcloned into the pET102/D- TOPO® (Life Technology) using a 0.5:1 molar ratio of fresh PCR purified product:TOPO® Vector in the TOPO® cloning reaction.

One Shot® TOP10 chemically competent cells of *E. coli* was gently mixed with 3 μl of the TOPO® cloning reaction, incubated on ice for 10 minutes, heat-shocked for 30 seconds at 42°C and immediately transferred to an ice bath. Then, 500 μl of of SOC medium was added to the reaction and incubated at 37°C for 1 hour (with shaking). An aliquote (100 μl) of bacterial culture was spread on a prewarmed selective plate (LB medium supplemented with ampicillin at 100 μg/ml) and incubated overnight at 37°C. Resistant colonies (transformed) had their plasmid DNA extracted and subjected to sequencng using both M13F and M13R primers independently, in an ABI 3500 Genetic Analyzer.

#### 2.4.3. *Protein Expression and Affinity Purification*

*E. coli* BL21(DE3) cells (Novagen) were transformed with the confirmed pET102/DeHys construction and selected on semi-solid LB medium supplemented with ampicillin (100 μg/ml). Bacterial cultures (OD600 of 0.4–0.6) were induced for 4 hours in the presence of 0.5 mM isopropyl b-D-1-thiogalactopyranoside (IPTG) at 30°C for for 4 hours. The cell pellets were resuspended in 50 mM phosphate buffered saline, 300 mM NaCl, 20 mM imidazole with SigmaFAST Protease inhibitor Cocktail (EDTA free) at pH 8, and lysed by three alternate freeze-thaw cycles, each cycle followed by sonication for 10 seconds with 60% amplitude. The lysate was centrifuged and the supernatant applied to pre-equilibrated $Ni2^{+}$-IMAC chelating columns (GE Health-care) following the manufactor's instructions. The protein was eluted with 200 mM imidazole. The eluted fraction (2 ml) containing *DeHys* was pooled and dialyzed against 200 ml of 20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM CaCl2 buffer overnight at 4$^{o}$C.

#### 2.4.4. *Western Blot*

Samples from all purification steps were first applied into a Tricine SDS-PAGE 10% gel and transferred to a PVDF membrane using a semi-dry blotter. The membrane was blocked with 5% low-fat milk solution and incubated in the presence of monoclonal anti-polihistidine antibody from mouse (1/3000) conjugated with alkaline phosphatase from (Sigma Aldrich). After washing the membrane with TBS-Tween three times, the membrane was developed using the BCIP®/NBT (Sigma) substrate system alkaline phosphatase colorimetric method.

#### 2.4.5. *Recombinant Protein Digestion*

The recombinant protein was cleaved with enterokinase and factor Xa protease (New England Biolabs Inc.). Digestion reaction was carried in 20 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM CaCl2 digestion buffer, under room temperature overnight. DeHys digestion patterns are shown in Table 1.

## 3. RESULTS AND DISCUSSION

### 3.1. *Nucleotide Sequence Analysis*

Through the use of heterologous PCR (a PCR using primers designed using a sequence from an organism different from target) using primers designed by Padovan et al. [13] for the EST TC147 from *P. vulgaris*, it was possible to amplify a genomic region consistent with a defensin from *E. hyssopifolia* (DeHys). Employing the software fGenesh, it was possible to describe the organization of the genomic region amplified. As expected, the sequence displayed two exons (with 64 bp and 173 bp, respectively to Exon 1 and 2) interrupted by one intron, which was responsible for the additional 124 bp, once the ESTs present only the protein coding region (Figure 1). The intron localization, inside the signal peptide sequence, is consistent with the described to others defensins obtained from genomic sequences, as *S. officinarum* [29], *V. unguiculata* [30], *Nicotiana megalosiphon* [31] and *Stellaria media* [32].

The coding region of gene DeHys was aligned against GenBank (NCBI), revealing some similarities to EST sequences from V. unguiculata (98%) and P. vulgaris (93%) corresponding to the sequence of DEF_VIGUN sequence (gi|225548305) [13] and to the sequence employed in the design of primers (gi|312982411). Similar data were found to *S. media*, which presented 94% of identity with other plant defensins sequences [32]. The coding sequence of DeHys also showed 93% similarity with the coding regions of a protease inhibitor of *Glycine max* (gi|255627362).

Additionally, members of the Euphorbiaceae family showed similarities of 80%, 79% and 75%, respectively, with *A. cassava* 4.1_020555m.g protein (of *Manihot esculenta*), low-molecular-weight-cysteine-rich proteins (of *Jatropha curcas*; gi|223469636) and of *Ricinus communis;* gi|255545389), respectively (data no shown). This pattern of similarity observed in Fabaceae members (considered moderate, compared to those obtained for some members of Fabaceae) may be attributed to smaller number of defensins of Euphorbiaceae family deposited in data bank and the amount and diversity of such molecules present within the various plant species. Although genome and transcriptome sequencing projects have been developed, leading to a rapid expansion of knowledge about the proteins [33], in this point of view, the family Euphorbiaceae is still lagging behind other traditionally cropped [34, 35].

### 3.2. *Structural DeHys gene organization*

The availability of complete genome sequences of eukaryotes allows us to address fundamental questions about the evolution of the structure of introns and exons in the structure of a given gene. Thus, we compared the structural organization of the gene DeHys with the six orthologs with greatest similarity, though most of them have not been described as defensin.

More often, the defensin protein precursor is composed of an address signal sequence to the endoplasmic reticulum, followed by a domain of mature defensin. Typically to defensins, the first exon encodes the signal

peptide almost entirely. The sequence is interrupted by an intron of variable size, followed by the second exon encoding the central domain of defensin [36, 37, 38, 39, 40, 41]. Our results were not different for the DeHys gene, with the same pattern found for plant defensins from other species, such as *V. unguiculata* [13] and *S. media* [42].

Another aspect of the analysis of gene structure referred to the size of the first exon, 64 bp which appears to be maintained, regardless of the family (Figure 2A). There was no conservation with respect to the size of the intron, however, the size of the second exon was maintained for *Manihot esculenta* and *R. communis* (170 bp), even though they are from distinct subfamilies of Euphorbiaceae (between each other and with respect to *E. hyssopifolia*) the *E. hyssopifolia* showed a triplet of nucleotides more, with 173 bp. This difference reflects the insertion of an amino acid at the end of these signal peptides regions (Figure 2C).

The intron consensus sequence, (A/C)AG|GT(A/G) are considered donor splicing site, while CAG|G, are acceptors. This pattern held for all Euphorbiaceae analyzed and almost all others, except *Petunia inflata* (gi|499654). In general, these sequences are conserved, but when the intron size varies, the region linking introns and exons are hotspots for indels. Curiously, this often occurs in most signal peptide regions, at the terminal portion, contributing to the diversity of the cleavage site and thus to the development of new defensins with terminal amino acids diversified. In the alignment shown in Figure 2C, it appears that the regions that most suffer from this kind of adjustment are located after amino acid 21. Thereby, the fragment comprising amino acids 1-21 correspond to the codons of the first exons.

Adaptive evolution of genes and genomes is the mainly responsible for the morphologic, behavioral, and physiological adaptation, and for the evolutionary divergence and innovations of species. To understand the role of natural selection, protein coding sequences enable to distinguish synonymous from non-synonymous substitutions, the last one changing the primary sequence of the encoded protein and is more likely to influence the fitness of an organism than a random replacement synonym that leaves the amino acid sequence unchanged [43, 44, 45]. Therefore, the comparison of the number of synonymous and non-synonymous (dS and dN) nucleotide replacements provides a powerful test of the hypothesis that the positive Darwinian selection has acted to accommodate change at the amino acid level [45]. Figure 4 shows three graphs where comparisons of dN against dS are plotted: sequences encoding the pro-peptide (hole CDS), the first exon (which encodes the signal peptide) and the second exon (encoding the mature defensin). By analyzing the average substitutions per site considering all sequences, the ones corresponding to the pro-peptide exhibited values of 0.180±0.026 and 1.032±0.086, to dN and dS, respectively, and in the sequence corresponding to the first exon, dN=0.245±0.053 e dS=0.782±0.100, while for the second exon, dN=0.128±0.030 and dS=1.010±0.099.

The ratio of the taxes of non-synonymous / synonymous substitutions, $\omega$ = dN / dS, has been widely adopted as a measure of selective pressure [46, 47]. When we tested statistically to purifying selection the pro-peptide CDSs, the signal peptide and the mature peptides domains, results were significant to negative selection (z-test, P <0,05), with values of 1,10572E-16, 2,39908E-06 and 8,41343E-15, respectively.

Genes involved in host defense often display high levels of genomic divergence and evidence for adaptive evolution [48]. Yet, studies in molecular evolution of genes from plants defense system are very limited [49]. What seems to happen to DeHys is that this gene may be conserved due its performance as a key actor to a proper organism function. For highly conserved genes, there is no extra gene copies in which the evolution can act and significant changes in this gene tend to be lethal, unlike the observed in the species mentioned above.

Thus, it seems more likely that a result significantly purifying may be indicative of a recovery from a prior positive selection [50], since data show that in general, antimicrobial peptides exhibit moderate disseminated positive selection [51]. Also according to Tiffin & Moeller [49], further studies that combine molecular evolution analyzes with ecological genetics should help to reveal how natural selection has outlined the evolution of plant defense genes.

### 3.3. *Similarity and Function*

The deduced amino acid sequence of DeHys consists of 78 residues, with the 31 amino acids of the N-terminal region corresponding to the probable signal peptide (Figures 1 and 2C). Thus, the program Philius Transmembrane Predictor program determined the position of the cleavage site between amino acids 31 and 32, and also showed that the signal peptide site indicates the secretion of the protein, addressing it to the endoplasmic reticulum. The same program identified the mature peptide sequence. This domais were composed by 47 amino acids, in accordance with the indicated to plant defensins (45-54 amino acids) [53]. Surprisingly, the DeHys propeptide sequence exhibited 100% of similarity with DEF_VIGUN [13]. Through alignment and similarity of the propeptide sequences (Figure 2B and 2C), we notice that other species closer to *E. hyssopifolia*, as *G. max* and *Medicago truncatula*, differed from it in two and four amino acids, respectively. However, such similarity can be explained by the large amino acid sequence convergence found at the various classes of antimicrobial peptides.

The levels of similarity between the mature peptides are usually higher when compared to values obtained in the analysis of ESTs, as observed by Slavokhotova et al. [32]. Blast search of the DeHys mature peptide against protein database (Figure 3A) revealed high similarity, between 100% and 80%, to "defensin" [of *V. unguiculata* (gi|225548306), *P. vulgaris* (gi|312982412), *Solanum pimpinellifolium* (gi|133711827), *S. lycopersicum* (gi|300827243), *Prunus persica* (gi|28624546) and of *Helianthus annuus* (gi|11387188)]; 83% - 78% similarity to "γ-thionins" (defensin synonym) of *Castanea sativa* (gi|16225423), *Solanum chacoense* (gi|170773916) and *Nicotiana tabacum* (gi|7939581), as well as 91% and 78% similarity to protease inhibitors of *G. max* (gi|533692) and *S. tuberosum* (gi|212525378), respectively. Two sequences of Euphorbiaceae (*R. communis*, gi|255545390 and *J. curcas*, gi|223469637) also showed similarity of 76% and 74%, respectively, to "low-molecular-weight cysteine-rich proteins". It is also important to note that all sequences above mentioned (like all the others included in the alignment) presented the same number and conserved cysteine spacing pattern of defensins (Figure 3B).

As DeHys and DEF_VIGUN [14], the protein BcAF from *Brassica rapa* demonstrated 100% similarity with a defensin from *Brassica napus*, with decreasing values for species from other genera [1]. However, in this work, related species are not so close. On the other hand, a high homology appears to be necessary since most of these peptides are dependent on interactions with lipid membranes, which limits the variability of the cysteine-stabilized alpha-beta (CSαβ) motif. Such architecture is characterized by three β-sheets and a short antiparallel α-helix, having four disulfide bridges located between the helix and the sheet, form the cysteine-stabilized alpha-beta (CSαβ) motif [54, 55], the conserved domain of the γ-thionins family (http://pfam.sanger.ac.uk//family/PF00304).

Regarding the putative function of DeHys, it was not possible to infer it by homology as the most similar amino acid sequences (disposed in Figure 3) are derived from nucleotide sequences, from which the function

was deduced trough the analysis of the expression induction patterns of these proteins and the resistance to determined biotic or abiotic factors resultant from them. Also, the transcripts described, are important in several activities, such as in the defense against fungi, bacteria, viruses; after processes such as physical injury, drought, temperature drop; in flower and fruit development, and maturation process, etc. (Figure 5C).

The similarity of peptides that belong to classes alternatively called defensins or protease inhibitors suggests that these plant peptides are characterized by the multifunctional activity and can act in defense against microorganisms or insects, as may be involved in the activation of other stress-responsive mechanisms. Such applications are consistent with the characteristics and functions described in the literature for *E. hyssopifolia*, which is used as antifungal [9, 54], antiprotozoal [9], antibacterial (Gram positives and negatives) [55], antiviral [6, 7], larvicide for insects [56], and has in its latex a large amount of proteolytic enzymes with inhibitory activity [57]. Pintus et al. [58] tried to relate the biochemical and protein composition of the latex of species from genus Euphorbia to its complex interactions in complex environments. Besides being resistant to environmental disturbance, inhabit in environments as diverse as the sandbanks [59] and the caatinga [60]. Such adaptability, favors its high invasive potential and consequent damage to agriculture [61], but also minimizes the damaging effects of pests competing with the agriculture for the pathogens [62], which predisposes this species to have different defensins.

### 3.4. *Structural organization*

The CSαβ motif is capable of supporting a wide variation in its primary amino acid sequence, although comparisons between defensins sequences from different plant species have revealed that this family has a clear, but limited, sequence conservation [3, 63, 64, 65]. About the composition of the protein, DeHys can be characterized as the described for PDEF_VIGUN [13]: considering the amino acid numeration of the mature peptide, DeHys features the characteristic amino acids from plant defensins, as the serine at position 7, the aromatic residue at position 10, two glycines at 12 and 32, a glutamic acid at 27, and the eight cysteines that provides the typical pattern of disulfide bridges (Figure 3B). These residues are conserved, probably because they have an essential role in folding or in stabilizing the protein [64].

For DeHys, there is a confirmation of some observations made by Padovan et al. [13] regarding the conservation of residues $Ser_7$ e $Glu_{27}$, which are explained by the fact that, although polar, they are internalized in the structure and form a network of hydrogen bonds essential to the skeleton establishment of the defensin, while the conserved residues from the positions 10 and 29 and the $Val_{23}$ contribute to the molecule core. The $Gly_{12}$ appears in a key position in the loop, connecting the first β-sheet and the α-helix (see Figure 3B), with its amide and carboxyl at the central chain making a network of hydrogen bonds, together with other conserved residues $Phe_{10}$ and $Arg_{40}$. The $Gly_{32}$ is subsequently positioned, overlapping the α-helix. In addition, other conserved residues in DeHys may have important structural role. $Asn_{19}$, for example, is involved in a network of hydrogen bonds that may indicate its action as a shield that stabilizes the α-helix, also involving the $Ser_{16}$. The $Leu_{42}$, that substitutes the $Phe_{42}$ present in all the other closer species, is not positioned near the core of the molecule, but projected from the β-sheet platform.

The secondary structure was composed of sheets β1 (from residue $Thr_2$ to $Gln_6$), β2 (from $Gly_{31}$ to $Cys_{34}$) and β3 (from $Cys_{41}$ to $His_{46}$), besides the α-helix ($Asp_{17}$ to $Thr_{26}$). About the four disulfide bridges, these were formed between cysteine residues of $Cys_3$-$Cys_{47}$, $Cys_{14}$-$Cys_{34}$, $Cys_{20}$-$Cys_{41}$ and $Cys_{24}$-$Cys_{43}$. The dendrogram

reveals high similarity between amino acids sequences, compared to the dendrogram of nucleotide sequences (Figure 3A). From the aligned peptides we also verify the major proportion of hydrophobic groups and polar cationic groups in comparison to the anionics, as seen in DeHys. It also appears that the similarity is not directly linked to the taxonomic organization of species. This fact is probably due to the variable number of antimicrobial peptides present in the species. So more than one defensin should be responsible for the defense of each species and few are available in the databases, as discussed earlier about the Euphorbiaceae.

Furthermore, as showed in Figure 3 from the alignment of 18 sequences available on the NCBI database most similar to DeHys (Figure 3A), it turns out that other residues were maintained such as: the Arg at positions 1, 38, 38 e 40, Ser at 5, Asn at 19, Ala at 21 and Phe at 29 (Figure 3B). Conserved domains beyond the essential for the defensins folding may have arisen as result of the limitations imposed by steric constraints of protein folding [66, 67, 68, 69]. Other studies have shown that the loop connecting β2- and β3-sheets is responsible for antimicrobial, channel blocking and α-amylase inhibitory activity [70, 71, 72]. At this site there is a need for positively charged amino acids so the peptide can be biologically active. Such pattern was detected in DeHys. Through site-directed mutagenesis in MsDEF1 revealed that the $Arg_{38}$ (numbering relative to MsDEF1 equivalent to the DeHys $Arg_{40}$) located in the loop between the β2- and β3-sheets is critical for the antimicrobial activity [71]. Other studies [72, 73], also emphasized the need of a positive residue in this region. De Samblanx et al. [74] showed that a variant of Rs-AFP2 ($Val_{39}$ Arg substituted corresponding to position $Arg_{37}$ of DeHys) presented greater antifungal activity. In DeHys, two more residues, $Arg_{38}$ and $Arg_{39}$, found in this loop region, preceded the $Arg_{40}$ (Figure 3B). These residues corroborate the suggestion that the defensin DeHys may have antifungal activity. The defensin So-D2 isolated from *Spinacia oleracea* leaves also presenting the fragment RRR (at the same positions 38,39 and 40) showed antifungal activity, besides acting against Gram-positive and gram-negative bacteria [75].

Curiously all defensins and/or protease inhibitors mentioned above display the $Phe_{42}$, while DeHys and PDEF_VIGUN have Leu replacing in this position (Figure 3B). It is also worth noting that there is a pattern of preference for positive residues in loop regions. In these areas, excluding positions with conserved residues, rare positions did not keep their chemical properties: the positions were 13, 28 and 30. These residues may be the main responsible for the type of function or the specificity of activity against a class of microorganisms, influenced by steric and electrostatic interactions of residues that may change while maintaining their properties. This observation correlates with the high variability in non-conserved sites and high rates of amino acid substitution that AMPs generally exhibit [76]. Therefore, it is remarkable the wide variation in the primary sequence of proteins aligned (Figures 2C and 3B). Despite the high variability, they all share a three-dimensional structure quite similar to that described in Figure 3, being the convergence of the tertiary structure the main relation to defensins identification [77]. Nevertheless, the lack of sequence homology makes it difficult to predict the activities of the peptides in vivo and makes it challenging to design potent synthetic antimicrobial peptides that have the desired activity in vivo [78].

### 3.5. *Protein Chemical Properties and Importance of the Signal Peptide*

From the data supplied by the properties predictors for the putative defensin DeHys, a comparative analysis between the pro-peptide and mature peptide revealed the importance of signal peptide (Table 2). Beyond the proper reducing the number of amino acids and thus its molecular weight, after the processing of the

peptide, the value retrieved (5,3 kDa) corresponded to the commonly found for plant defensins (approximately 5 kDa [79]). Moreover, the mature peptide has a higher theoretical isoelectric point (9,7), indicating an increase in the peptide acidophily after processing, which provides a greater potential to mature peptide perform its activity. Therefore, the signal peptide plays a role in addition to addressing the protein. Furthermore, the basic IP obtained, corresponding to usually found for the defensins mature peptide, with IP rounding 9 [3].

About charge of the molecule (Table 2), one positively charged residues and two negatively charged are lost, what increase the positive charge of the mature peptides. This positive charge is due to the presence of a large number of Arg and Lys residues (positively charged), comparing to Glu and Asp residues (anionic). This feature facilitates the peptide interaction and insertion into the cell wall and in the interaction with the anionic phospholipid matrix of the plasma membrane of microorganisms [64, 80]. In fact, the values obtained by prediction of hydrophobicity ratio indicate that the processing of the signal peptide promotes a decrease in the hydrophobic character of the molecule, increasing the affinity of the protein for the cell membranes. Simultaneously, the propeptide that was slightly hydrophobic (positive GRAVY index), comes to be slightly hydrophilic (negative GRAVY index), what favors some facility for transport in fluids. The signal peptide fragment is often acid and may also play a role as a suppressor protein until the maturation and activity of the mature domain defensins which follows. These proteins enter the secretory pathway and have no obvious signs of post-translational modification or directed to specific subcellular addresses. This prodomain can contribute to the maturation of the defensin, acting as an steric intramolecular chaperone and/or preventing deleterious interactions between defensins and other cellular proteins or lipid membranes, neutralizing the toxic activity during translocation through the secretory pathway [81, 82, 83].

Figure 4 shows that the more acidic the medium, higher the protein charge, increasing the potential for docking to the negatively charged structures, such as anionic membranes of microorganisms. This observation agrees with the accepted mechanism of action for antimicrobial peptides. The net charges of these molecules often vary considerably between -4.8 and +10.4, with variation of pH [84]. At physiological pH (5.5), DeHys present +6 charge. Other proteins, like PsDef1 e RsAFP2, showed +6.7 and +5.8, respectivamente [85, 86]. The opposite can be seen commonly to most fungal plant defensins, as well as other types of defensin which is the reduction of antifungal activity when the cationic strength of the medium is increased [85, 87, 88, 89]. This is due to the presence of cations, with divalent being at least one order of magnitude more potent than monovalent cations [85, 89, 90].

### 3.6. *Prediction of the tertiary structure and related chemical properties of DeHys*

Understanding the mechanism of a protein function generally requires the knowledge of its three-dimensional structure [91, 92], which is in last instance determined by its amino acid sequence [93]. The method of modeling by homology is one from which it obtains the three-dimensional structure of a given protein sequence based primarily on its similarity to one or more proteins of known structures [94, 95]. By searching the sequence of DeHys against the PDB database structures, the four molecules with the most similar sequence were: 1GPT (Hordeum vulgare), 1GPS (Triticum turgidum), 1MR4 (Nicotiana tabacum), and 1N4N (Petunia hibrida), with 53%, 49%, 45% and 40% of similarity, respectively. (All obtained by Nuclear Magnetic Resonance - NMR).

According to Sanchez & Sali [96], when sequence identity is greater than 40%, the alignment happens satisfactorily. These structures were, then, employed in the design of the multiple sequence model by VMD 1.9 program at Modeller 9v7, generating the three-dimensional structures (Figure 5). The peptide topology, as well as their various properties is showed in Figures 5 and 7. The prediction of structures of plant defensins by comparative modeling was used to characterize the 3D structure of other especies, such as Cp-thioninI and Cp-thioninII from Vigna unguiculata [97, 98], Gbd from Ginkgo biloba [99], VrD1 from V. radiata [100], Vv-AMP1 from Vitis vinifera [43] and PhaDEF from Phaseolus vulgaris [101].

In order to analyze the quality of the tertiary structure predicted to DeHys, this was subjected to a series of analyzes. The first, QMEAN 6 comprises a method of composite score of six linear descriptors using statistical potential [25]. As result, the 3D structure of DeHys obtained values of 0.43, indicating a good reliability of the model. In an analysis for a geometric quality measure, the z-score of a structure based on NMR, must be greater than -5, and preferably greater than -2 [26], the value obtained (-1.16), shows the accordance of the modeled protein with the desired quality standards (Figure 5A). The distribution of the amino acid residues in the Ramachandran plot, which evaluates the stereochemical quality of the structure, revealed that 90% of the residues were located in the most favored regions for a good quality model (Figure 5B). In molecules identified through NMR, at least 85% of the residues should be plotted in these regions [27]. Finally, G-factor evaluates how unusual the stereochemical properties of the residues. The value obtained for the model DeHys was -0.07. In general, the properties are good if greater than -0.5 [27]. Therefore, from four different parameters, we verified the reliability of the model designed for the putative defensin from E. hyssopifolia.

The Procheck also provided data about the relationship between the amino acid sequence and the residues positioned in disadvantaged regions of the Ramachandran plot (Figure 5C). These residues refer to the Arg38 and Arg39, potentially involved in the antifungal activity. If we look at the structures are available in PDB used in the analysis (1GPT, 1GPS, 1MR4 and 1N4N), the four exhibit the Arg39 and Arg40. The non-favoring observed for these residues may be due a possible steric hindrance, caused by the positive ionic charge repulsion between the side chains in the fragment RRR. About the relationship between sequences, secondary structure and accessibility estimated (Figure 5D), the 3D data coincided with obtained by ESPript2.2 about the secondary structure, while the accessibility of the residues showed that the edges N- and C-terminal and the looping areas are more accessible compared to the compact structure located in the CSαβ core. In the loop region between sheets -β2 and -β3, we verified that the Arg38 and Arg39, demonstrated some accessibility. In this area there are probably the favoring of molecular interactions promoted by high concentration of residues with long and cationic side chains (from the six amino acids that compose de four are arginines), to which is allowed a limited flexibility, showing an important role in changes in the conformation of the active site. On the other hand, patterns obtained by NMR are solution-like structures, what means that they present the same conformation in solution, since the model takes into account the effects of the medium, different from structures obtained via X-ray crystallography, where protein are prepared as salts. Given the above, the structure of DeHys presents itself in a natural conformation, in which the peptide is active, with some slight variations possible (limited by the rigidity of the CSαβ motif) resulting from electrostatic interactions with medium components or other macromolecules. Thus, this could be probable conformation likely present in drug dosage forms liquid or semisolid based.

From Figure 6A-D, we found that some spatial characteristics were evidenced by the predictors. The arrangement of amino acid residues according to polarity and charge showed that DeHys is quite cationic, with an arrangement of basic residues are clustered at the bottom and the top of the structure, predominantly in the loop regions connecting the β-sheets and the α-helix. As already discussed, the protein displays a slightly hydrophilic character, which justifies its polarity, despite the presence of numerous hydrophobic residues. It is also verified that the disulfide bonds located inside the structure, exerting its effect to stabilize the architecture CSαβ. Furthermore, the molecular surface characterization of the protein through software DeepView v4.04 allowed inferring about the accessibility and the exposition of regions and side chains of the molecule (Figure 7A) as well as about its electrostatic potential (Figure 7B), important in the interaction with membranes. Such analysis revealed the high electrostatic potential predominant in the regions more accessible of the molecule, coupled to the cationic characteristic of most of these fragments, as already observed for defensins from P. vulgaris [101] and V. unguiculata [102].

When they reach a threshold concentration, cationic peptides accumulate on the membrane surface. After the initial electrostatic interactions with negatively charged components of the membrane, these peptides (which are positively charged) are inserted in the membrane and organized together to form ion channels [73], or even interrupt the continuity of the membrane trough the subsequent neutralization of this lipids, allowing the passage of various molecules, from ions to proteins [13, 29, 103]. Thus, the ability of cationic peptides in general to permeabilize the cytoplasmic membranes may be a mean to hit an intracellular target [104, 105, 106]. The positively charged residues may also interact with the lipid membrane via specific receptors on the cell surface [107, 108], consequently, the peptide binding to the membrane can activate various pathways that may cause cell death.

### 3.7. *Heterologous expression*

Heterologous expression approaches have been widely used in onder to produce large amounts of purified functional proteins for medicinal and biotechnological application [109]. In this sense a transformed E. coli expression strain baring the DeHys gene inserted in an expression vector was required.

One Shot® TOP10 resitent colonies were tested for the presence of the synthetic pIDTSmart-Amp-DeHys plasmid. Positive colonies had their plamidial DNA extracted and submitted to a new PCR using the primers designed to prepare the mini gene for subcloning in the expression vector. The Phusion High-Fidelity DNA Polymerase PCR product showed a length of 160 bp, while the DeHys gene alone was comprised of 144 bp coding a 48 aa peptide (Figure 8). This length increment was due to the TOPO® overhang (“CACC”) needed for blunt-end cloning in the pET102/D- TOPO expression vector and due to the factor Xa sequence (12 bp) positioned downstream the gene in order create a way to eliminate the polyhistidine tag from the fused protein.

When analyzing the results of the construct nucleotide sequencing it was possible to confirm the first DeHys codons, starting with “ATG” (Met) preceded by the “cacc” overhand adapter for correct pET102/D-TOPO cloning (Figure 9A) and also DeHys final codons followed by the Factor Xa site sequence (Figure 9B). These findings led to the confirmation that the gene sequence was amplified correctly and the amplicon was cloned in the right frame on the expression vector.

In order to check the capacity of transformant *E. coli* to solubly express our protein, a preliminary expression assay was performed. Total crude cell extracts of induced and non-induced cultures were submitted to SDS-PAGE electrophoresis and as expected a strong signal/band was only present in the induced sample.

Such band presented an approximated 24 kDa profile. A very similar molecular weight was predicted for the fused protein when feeding the Molecular Weight Calculator tool from www.bioinformatics.org with the translated ORF from the expression vector bearing the DeHys gene. The predicted protein was of about 23.5 kDa (Figure 10).

Using the same tool we were also able to determine the predicted contribution of the diverse elements of the ORF in the heterologously expressed fused defensing. Only one fifth (5.3 kDa) of the predicted molecular weight would be represented by DeHys, while the fused thioredoxin would be responsible for approx. half of it (11.9 kDa). Other elements (V5 epitope, EK and Factor Xa restriction site) and sequences belonging to the expression vector, presented up and downstream the defensin gene, would sum up to achieve the final 24 kDa profile.

With the aim to further check the identity of our purified expressed protein, samples were submitted to proteolitic cleavage with enterokinase and/or Factor Xa. The digestion pattern of the studied recombinant defensing was used as a second checkpoint. When digested with enterokinase, all expected fragments were obtained. An extra band represented by the enterokinase itself (approx. 31 kDa) was also observed. The digestion with factor Xa also produced all predicted fragments (3.4 KDa and 20.2kDa), plus two reduced factor Xa subunits bands of 30 kDa and 16 kDa (Figure 11) (citar a referencia do manual do fabricante)

When performing a double digestion with both proteases, just the two heavier expected bands were present (band of 12.8 kDa representing the thioredoxin and a 6.7 kDa band representing the DeHys + Epitope V5). The third band of 3.4 kDa may not have been detected due to its low concentration (Figure 11). Such experiment had a great importance, as isolating the defensing DeHys from its fused thioredoxin and polyhistidine tag is crucial when thinking of testing it for its biological activity or therapeutic use.

As a result from the purification procedure, we were able to elute a single band of 24 kDa on SDS-PAGE gel (Figure 12A.) To check for the presence of the intentionally added polihystidine tag in our recombinant DeHys, the eluted protein was submitted to a Western blot analysis. The western blot was positive i.e. showing that the purified protein was recognized by the anti-polihistidine antibody, enhancing our certainties of having successfully expressed the construction TRX-DeHys-6xHis (Figure 12B).

In order to use DeHys in future biological activity experiments and to double-check it's correct expression, it would be necessary to remove the fused TRX and the 6xHis tag from it's N and C terminus, respectively. Eliminating the TRX from the DeHys and from the solution prior the biological activity tests is essencial for an accurate evaluation. The human β–Defensin 1(hBD-1) usually presents a minor antibiotic killing activity, but after been reduced by TRX, hBD-1 becomes a potent antimicrobial peptide against the opportunistic pathogenic fungus *Candida albicans* and against anaerobic, Gram-positive commensals of Bifidobacterium and Lactobacillus species [110].

## 4. CONCLUSIONS

This study identified and characterized a new defensin coding gene from a wild species as E. hyssopifolia, analyzing aspects such evolution and gene organization of this class of peptides and checking their

conservation among sequences of other plant defensins already described. It is interesting to notice that both the nucleotide as the amino acid sequences from this new defensin positioned themselves more closely to other defensins described for Fabaceae, probably due to lack of genomic and proteomic studies in the Euphorbiaceae family, besides the complex relationships that leads this molecules to evolve, showing not correlated with the phylogenetic organization of the species involved. Yet, the tendency to negative selection observed demonstrates the importance of high conservation at the analyzed sequence to plant defense. The putative defensin DeHys obeys to the cysteine-stabilized alpha beta (CSαβ) motif and presents the characteristic conserved residues, with changes in the other positions being responsible by the interaction with the membrane. Also, a recombinant defensin fused to TRX was also obtained through heterologous expression. Although it is still necessary to test the expressed DeHys for its supposed biological activity specially its antimicrobial activity.

Therefore, the comparative modeling of the three-dimensional structure and prediction of its chemical properties, such as the molecule folding and visualization of residues placement; molecular weight; theoretical isoelectric point, total net charge, charge under pH variation; amphipathicity; binding potential; in addiction to estimated half-life, are together parameters of outstanding importance for the understanding of this type of molecule, providing the first step to the development of heterologous expression approaches, and also assisting to the design of new products for therapeutic or agro-biotechnological purposes.

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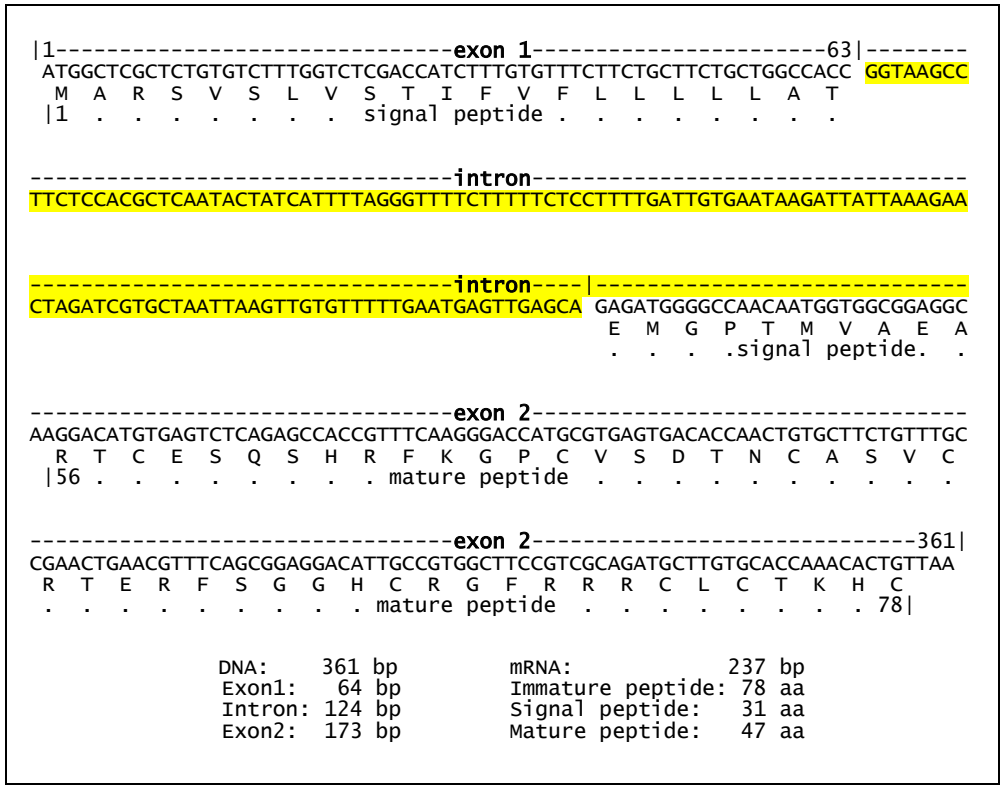
**Table 1:** Expected fragments sizes of the recombinant DeHys after proteolitc digestion with enterokinase, fator Xa and enterokinase+fator Xa (double digestion).

| **Proteases** | **Weight** | **Composition** |
|---|---|---|
| Enterokinase | 1 – 10.1 kDa | 1 – DeHys + V5 Epitope + 6xHis |
| | 2 – 12.8 kDa | 2 - Thioredoxin |
| Factor Xa | 1 – 3.4 kDa | 1 – 6xHis |
| | 2 – 20.2 kDa | 2 – Thioredoxin + DeHys + V5 Epitope |
| Enterokinase + Factor Xa | 1 – 3.4 kDa | 1 - 6xHis |
| | 2 – 6.7 kDa | 2 - DeHys + V5 Epitope |
| | 3 – 12.8 kDa | 3 - Thioredoxin |

**Table 2**. Prediction of chemical properties of pro-peptide and mature peptide from DeHys codifying gene. Items marked with "*" were predicted by APD2 predictor software, the remaining, by ProtParam software.

| **Features** | **Pro-peptide** | **Mature Peptide** |
|---|---|---|
| Number of aminoacids | 78 | 47 |
| Molecular weight (Da) | 8659,1 | 5365,1 |
| Negatively charged residues (Asp + Glu) | 5 | 3 |
| Positively charged residues (Arg + Lys) | 11 | 10 |
| Whole Charge* | +9 | +10 |
| Theoric isoleletric point | 9,16 | 9,37 |
| Hidrophobicity ratio * | 44 % | 31 % |
| *Grand average of hydropathicity index* (GRAVY) | 0,078 | -0,819 |
| Protein binding potential (Boman index)* | 1,83 kcal/mol | 3,58 kcal/mol |
| Aliphatic index | 68,72 | 22,77 |

**Figure 1.** Nucleotide sequence and predicted genes/exons and aminoacid sequence of the putative protein DeHys obtained through fGenesh and Philius. (Sequences hidden due to patente issues)



880 **Figure 2**. Estructural analyses of the gene and pro-petides similarity. (A) Heterologous
881 comparison of planta defensins. (B) Dendrogram (*Neighbor-Joining*, bootstrap of 1.000
882 replications) of pro-peptides and (C) alignment of the sequences. Species arranged in
883 decresing order of similarity against *Euphorbia hyssopifolia*: *V. unguiculata* (gi|225548305);
884 *G. max* (pz|Glyma16g18480); *M. truncatula* (pz|AC229689_3); *P. persica*
885 (pz|ppa014246m.g); M. *esculenta* (pz|cassava4.1_020555m.g); *S. pimpinellifolium*
886 (gi|133711823); *P. inflata* (gi|499654); *R. communis* (pz|29908.t000184).
887
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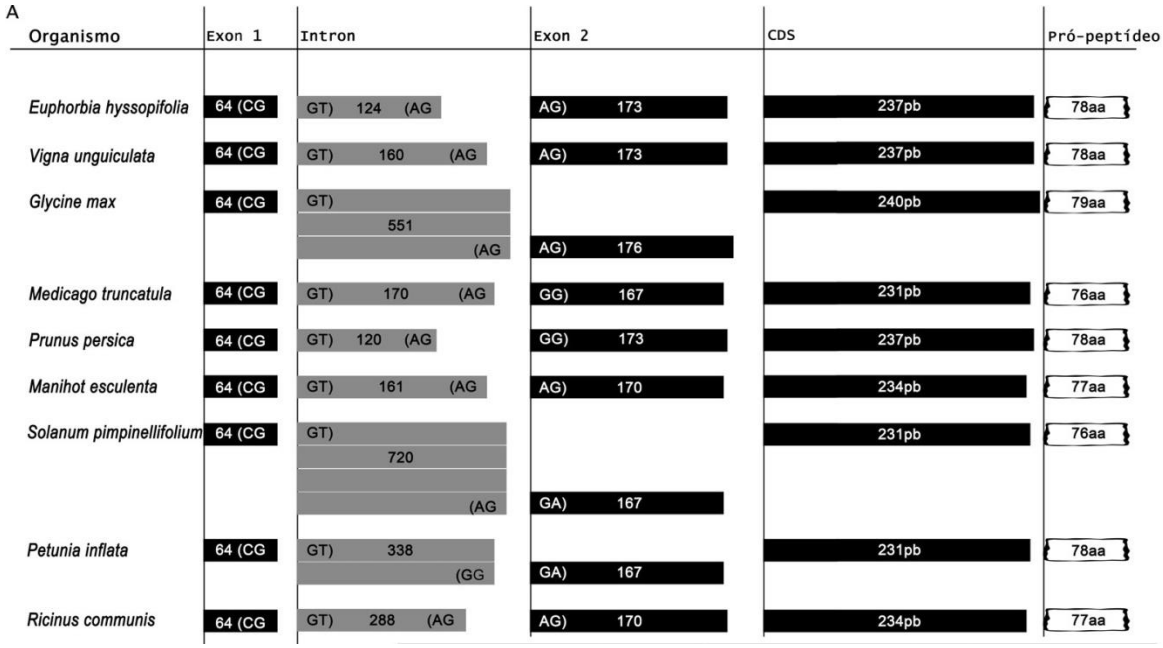


**Figure 3** Sequence analyses of the most similar mature peptides defensins. (A) Sequence dendogram by the Neighbor-Joining method with bootstrap of 1.000 replications on MEGA 5.05 program. (B) Alignment by ClustalX2 (Larkin et al., 2007). Hidrophobic residues are in blue, cationic in green and anionic in red. Numbers bellow the aligments indicate the formed disulfide bonds. Above the aligment are the predicted secondary structure.by ESPript 2.2 (Gouet et al., 1999). Disulfide bridges indicated by DISULFIND (Ceroni et al., 2006).

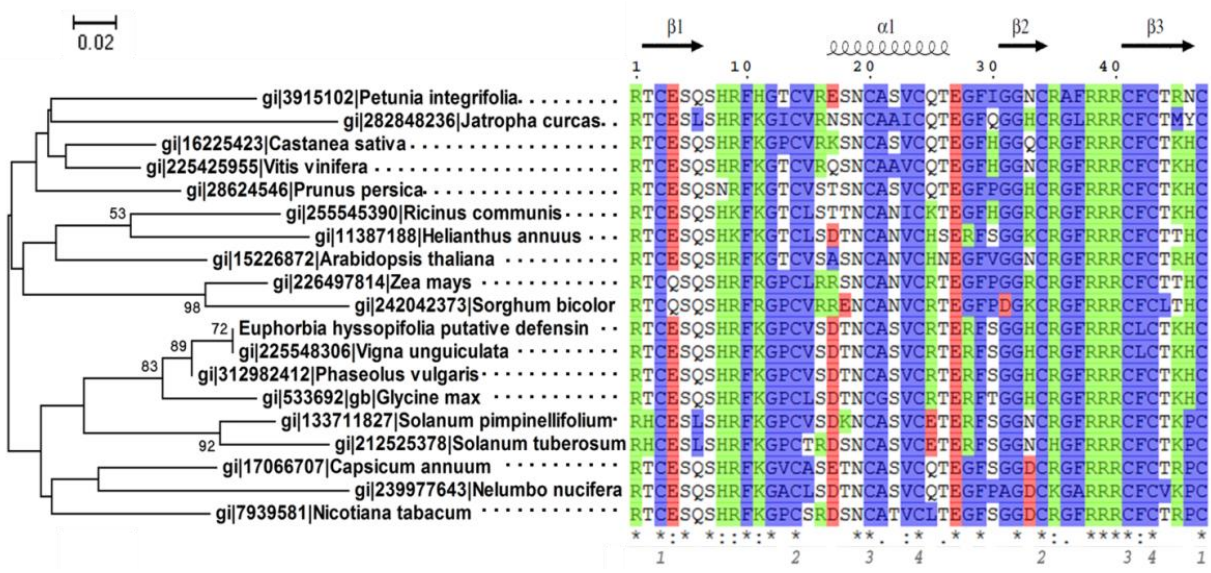


**Figure 4**: Estimated total charge for DeHys under pH variation. Values obtained through Protein Calculator v3.3

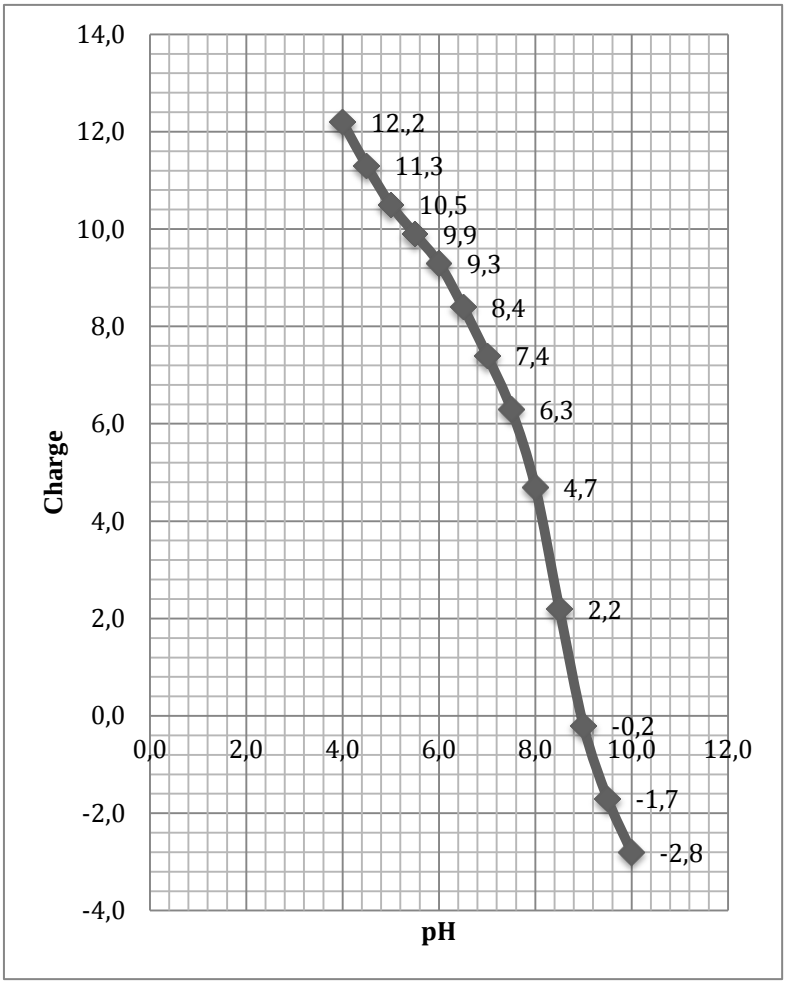


**Figure 5.** Tridimensional structure of DeHys defensin, showing the beta sheets architechture, alpha helix and the spacial distribution of amino acids residues. In (A) aa colored accordingly to each molecule acid-base feature, with positive residues (acids) in red, negative (alcaline) in blue and neuters in purple. In (B), accordingly to the polarity, polar residues in cyane , and in purple, apolars. Cisteins and disulfide bonds are represented in green. Structure obtained through VMD 1.9 and Modeller 9v7, edited at Jmol Viewer 12.0

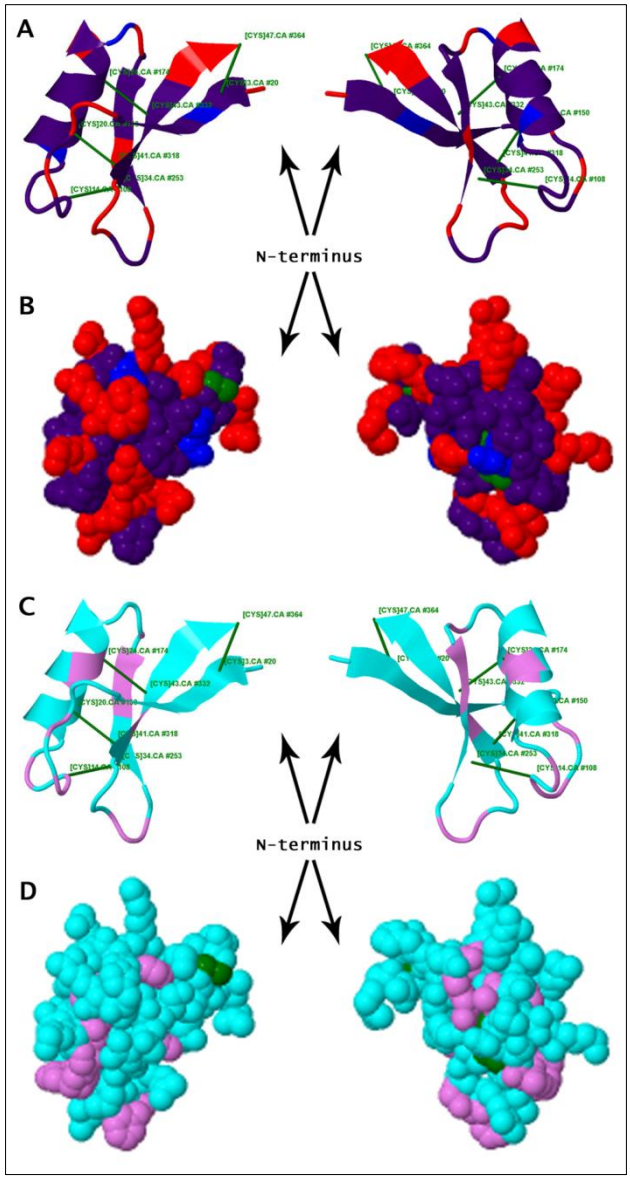


**Figure 6.** Evaluation of tridimensional structure quality for DeHys. (A) in red, the Z-score, expectedfor molecules elucidated through NMR. (B), Amino acids residues dispersion in Ramachandran plot. (C) Positioning by residues. (D) Accessibility by residues. (A) obtained through QMEAN6 global score; (B), (C) e (D), through PROCHECK v.3.5.4.

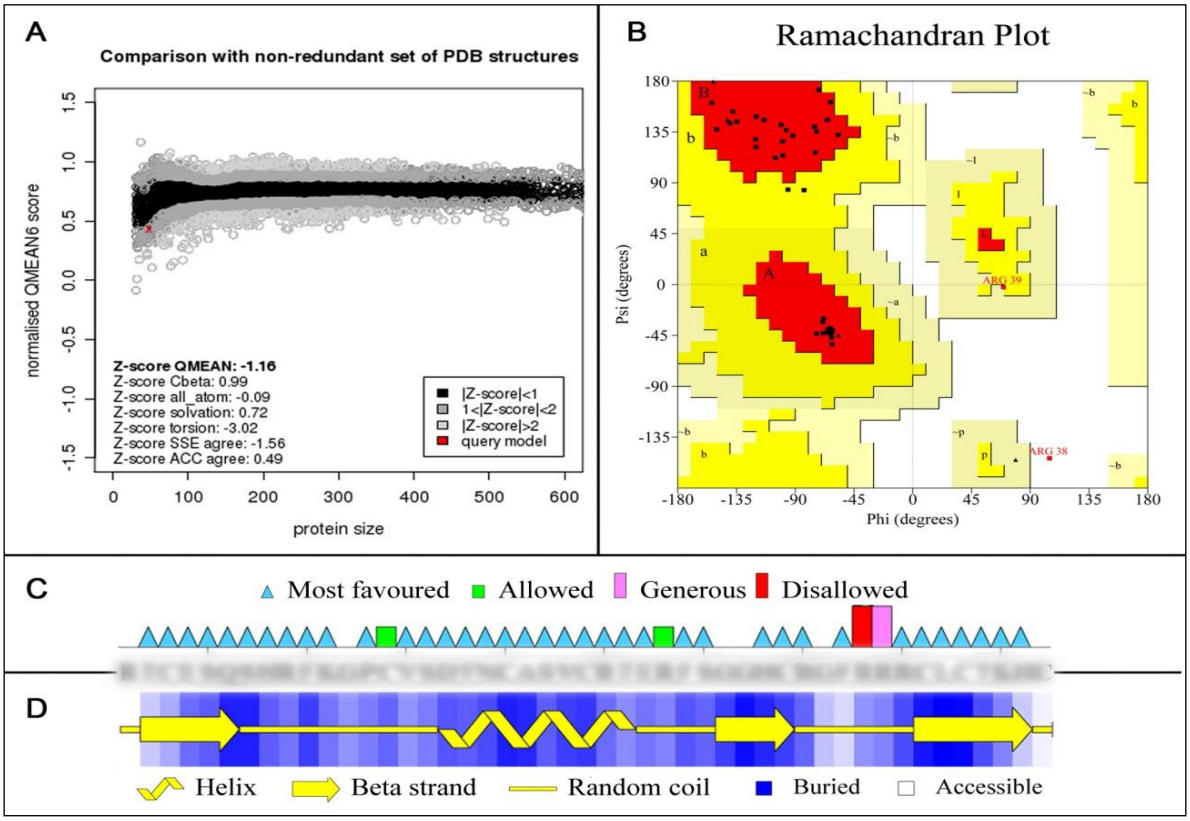


**Figure 7**. Tridimensional structure molecular landscape of putative defensin DeHys. In (A) colored accordingly to molecule accessibility (color pattern: warm colors, more accessible regions, cold colors, less accessible regions; in (B) molecule colored accordingly to its eletrostatic potential (blue and red, positive and negative eletrostatic potential eletrostático, respectively). Molecule obtained through Modeller 9v7 and characterized with DeepView v4.04. Eletrostatic potential obtained through Poisson-Boltzmann method.

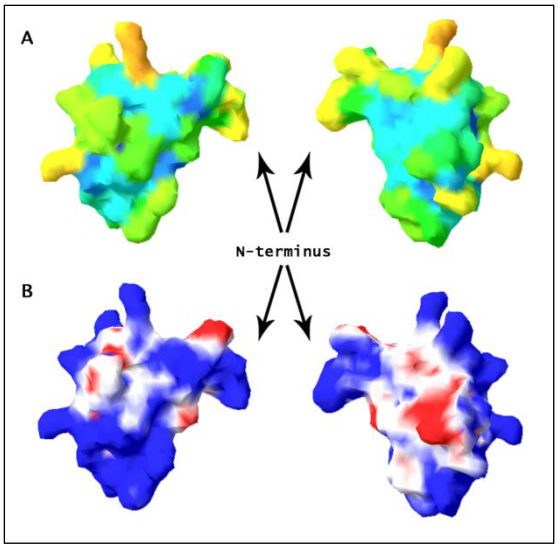



**Figure 8.** High-Fidelity PCR product agarose gel (1%). Lane 1: 100 bp ladder. Lane 2: amplicon *DefHys* with TOPO® overhang ("CACC") and Factor Xa sequence.

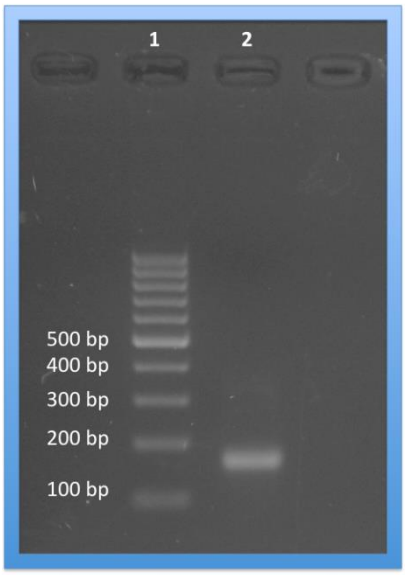


**Figure 9.** DNA sequencing of DeHys construction A) In red the first DeHys codons. B) In red the last DeHys codons and in green the Factor Xa site sequence.

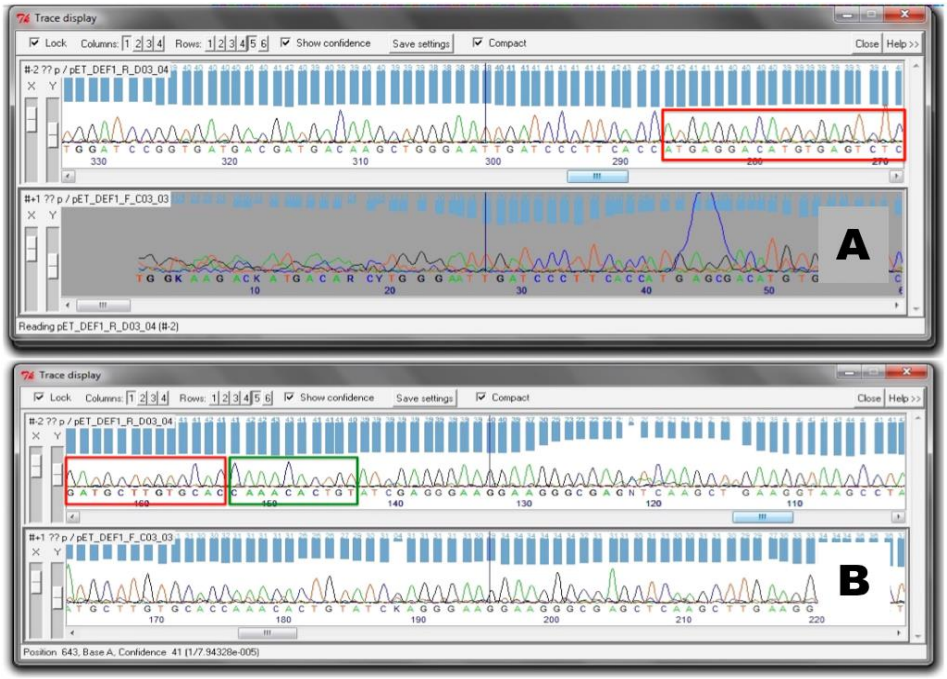


**Figure 10.** Preliminary expression assay SDS-PAGE gel (15%). Lane 1: not induced total soluble protein extract. Lane 2: induced total soluble protein extract. Lane 3: empty. Lane 4: Prestained Protein Marker.

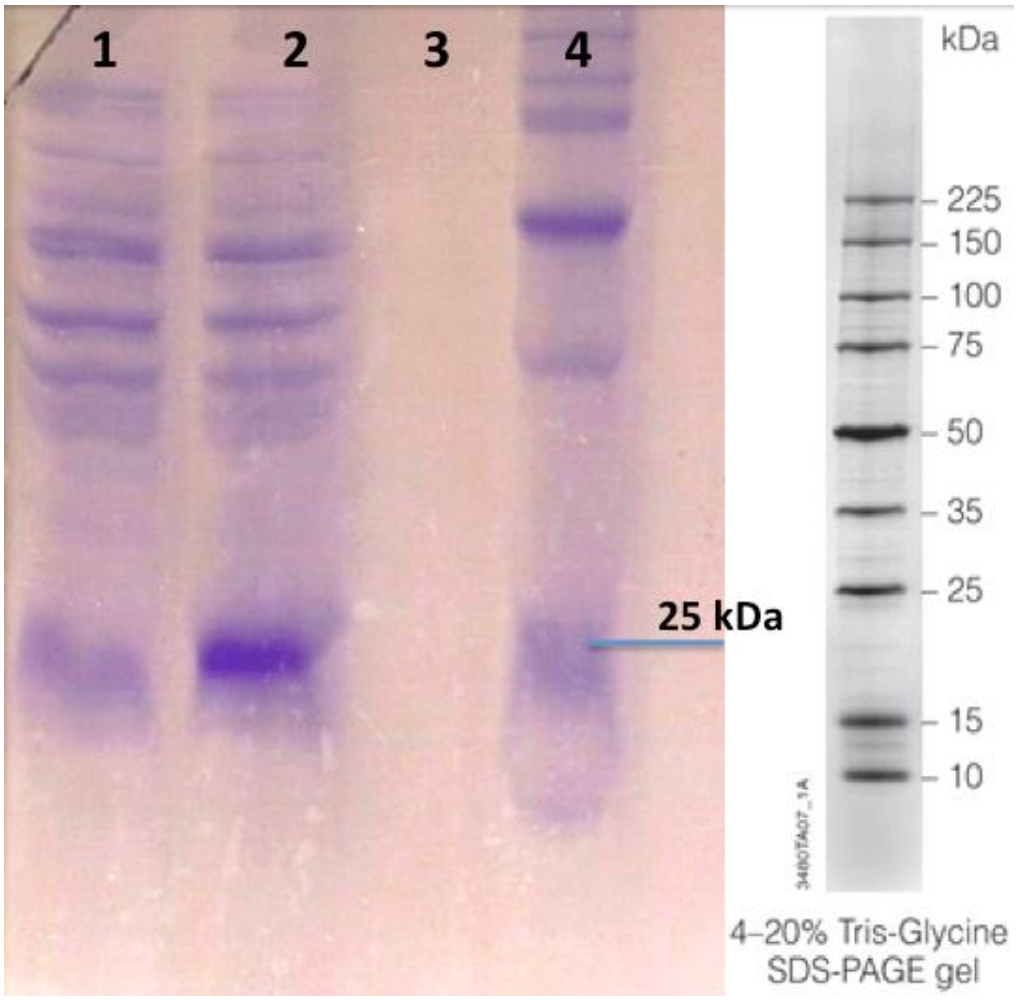


**Figure 11.** Digestion pattern analysis in Tricine-SDS-PAGE gel . Lane 1: prestained protein molecular marker. Lane 2: recombinant DeHys digested with enterokinase. Lane 3: recombinant DeHys digested with fator Xa. Lane 4: Double digestion of recombinant DeHys with enterokinase and fator Xa.

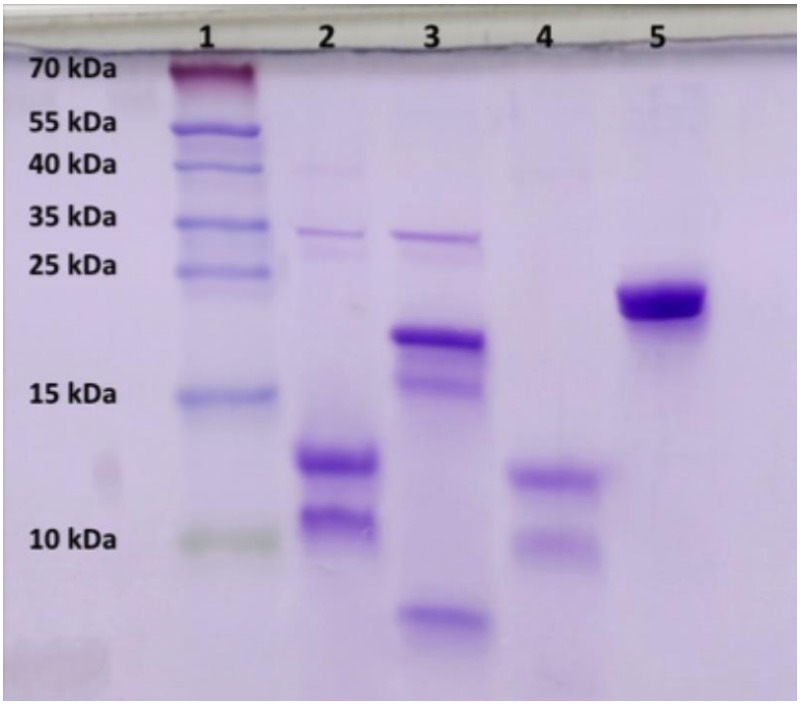
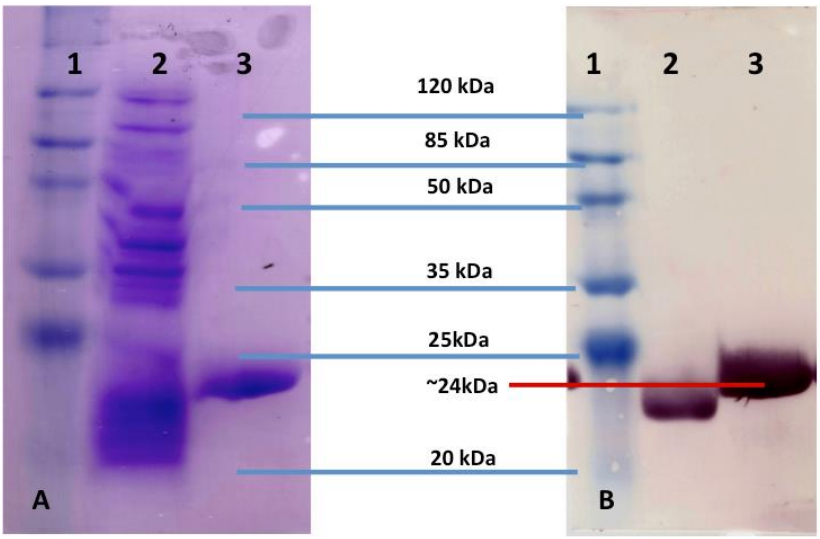



**Figure 12.** Results for the Ni-NTA column purification. A) SDS-PAGE gel (15%) and in B) the Western Blot. Lane 1: Prestained Protein Molecular Marker. Lane 2: Flowthrough. Lane 3: Eluted DeHys fused protein.



## 5 DISCUSSÃO

A aplicação de proteínas recombinantes, seja no campo da farmacêutica, , seja na agrobiotecnologia, depende de uma quantidade viável de material biológico. A purificação de proteínas vegetais de interesse comercial pode se mostrar algo custoso e complexo, tendo em vista o repertório altamente variado que uma amostra de extrato protéico vegetal pode ter. A utilização de proteínas exógenas para fabricação de medicamentos exige um alto grau de pureza, por essa razão, a utilização de sistemas de expressão heteróloga como biofábricas para a produção desses peptídeos tem demonstrado ser uma abordagem promissora (VRIENS et al., 2014). Com esse objetico, uma linhagem de expressão de *E. coli* foi transformada com um vetor contendo uma construção para um gene de defensina da *Euphorbia hyssopifolia*.

A análise do eletroferograma do sequenciamento de clones contendo a contrução, permitiu confirmar não só a presença do inserto, como também sua correta síntese e precisa posição de seu quadro de leitura. Antecedendo o sequenciamento, houve uma checagem preliminar através da reação em cadeia da polimerase (PCR) que demonstrou a presença de um produto de PCR com o comprimento de 159 pb. Apesar do gene de interesse ter apenas 144 pb, o fragmento encontrado conta ainda com um incremento de 15 pb devido a adições flanqueando o gene DeHys: adaptador “CACC” para clonagem no vetor pET102/D-TOPO e uma sequência para o sítio de clivagem pelo fator Xa posicionada de forma a possibilitar a eliminação da tag de polihistidina da proteína recombinante.

Bactérias podem armazenar substâncias tóxicas ou estranhas em uma forma insolúvel denominada de corpos de inclusão. Algumas estratégias priorizam os corpos de inclusão como forma de concentrar a proteína de interesse, porém essa abordagem exige uma etapa de solubilização o que aumenta a complexidade da extração do produto de expressão (PANTELEEV; OVCHINNIKOVA, 2016) . Ter sua proteína expressa de maneira solúvel no citoplasma bacteriano torna mais simples o processo de purificação. Para aumentar as chances de garantir a solubilidade de uma proteína em um sistema de expressão bacteriano, costuma-se adicionar proteínas de origem bacteriana fusionadas à proteína de interesse (GEUM et al., 2015). A thioredoxina foi usada na construção da DeHys recombinante com o intuito de burlar o processo de formação de corpos de inclusão e também impedir

que as defensinas expressas possam se aglomerar, formando poros na membrana bacteriana levando à morte a célula transformada (NGUYEN et al., 2011).

Um ensaio preliminar de expressão foi realizado, onde duas culturas de *E. coli* carregando o vetor contendo o gene DeHys foram submetidas à duas condições: a primeira cultura não teve sua expressão induzida e a segunda teve a expressão de DeHys induzida através da adição de IPTG ao meio de cultura. Foi possível observar, através da aplicação de amostras de proteínas totais solúveis de cada tratamento, em gel de SDS-PAGE 15% o aparecimento de um banda de sinal forte somente nas amostras oriundas das células induzidas, de modo que ficou constatado que a proteína recombinante havia mantido sua solubilidade.

A banda diferencial no teste preliminar de expressão apresentou tamanho compatível com o predito, para a proteína DeHys recombinante, através da ferramenta Molecular Weight Calculator tool" em www.bioinformatics.org.

A adição, no momento do desenho experimental, de sítios únicos para duas proteases (enterokinase e Fator Xa), nos permitiu não só confirmar a identidade da proteína purificada através do padrão de bandas após digestão proteolítica, bem como permitir o isolamento da defensina dos demais elementos da construção, como thiredoxina e tag de polihistidina.

Através do uso de uma coluna de Ni-NTA foi possível isolar uma banda única de tamanho aproximado de 24 kDa, a qual quando submetida a uma análise por western blot, mostrou-se positiva para a presença da tag de polihistidina, reforçando os resultados obtidos no teste de digestão por proteases.

## 6 CONCLUSÕES

O presente trabalho visou a expressão de em E. coli de uma defensina nunca antes descrita e codificada por um gene em Euphorbia hyssopifolia. Foram providos então, ao longo do trabalho, não só o conteúdo teórico, para aqueles que desejem trabalhar com expressão heteróloga de defensinas em E. coli (na forma de um artigo de revisão), bem como todos os procedimentos técnicos para a obtenção de uma defensina, descritos de maneira detalhada no Capítulo II.

Através da análise por eletroforese, ensaios de digestão por proteases e western blot, foi possível verificar a eficácia da estratégia de expressão, bem como da construção utilizada.

Embora durante o estudo, tenha sido obtida com sucesso a expressão heteróloga de uma defensina de *Euphorbia hyssopifolia*, nunca antes descrita e estudada, a mesma ainda precisa ser testada quanto à sua atividade biológica. Somente com os resultados dos testes de atividade biológica poderemos inferir sobre seu possível uso farmacêutico.

Assim sendo, esperamos nesse estudo conseguir auxiliar os leitores, no desenvolvimento e obtenção de novas defensinas, sejam elas para propósitos terapêuticos ou agrobiotecnológicos.

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# APÊNDICE – CURRÍCULO LATTES

CNPq — Conselho Nacional de Desenvolvimento Científico e Tecnológico

Currículo Lattes

Imprimir currículo

## Luiz Rodrigo Saldanha Gazzaneo

Endereço para acessar este CV: http://lattes.cnpq.br/1585982858539877

Última atualização do currículo em 11/02/2016

### Resumo informado pelo autor

possui graduação em Bacharelado em Ciencias Biologicas pelo Centro de Ciências Biológicas (2004). Pós-graduado (Mestrado) pelo departamento de Genética da Universidade Federal de Pernambuco. Tem experiência na área de Genética Molecular e Melhoramento Vegetal, atuando principalmente nos seguintes temas: biologia molecular, mutagênese, cultiura de tecidos. Apresenta também experiência no Ensino Superior tendo ministrado disciplinas como Genética Humana, Genética e Evolução, Microbiologia, imunologia e Biologia Molecular.

**(Texto informado pelo autor)**

### Dados pessoais

**Nome** Luiz Rodrigo Saldanha Gazzaneo

**Nome em citações bibliográficas** GAZZANEO, L. R. S.

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**Cor ou Raça** Parda

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### Formação acadêmica/titulação

**2011** Doutorado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Prospecção e validação de defensinas em plantas medicinais para o desenvolvimento de novos fármacos antimicrobianos
Orientador: Ana Benko-Iseppon
Bolsista do(a): Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
*Palavras-chave: peptídeos antimicrobianos, Expressão heteróloga*
*Áreas do conhecimento : Biotecnologia*
*Setores de atividade : Saúde humana e serviços sociais, Pesquisa e desenvolvimento científico*

**2005 - 2007** Mestrado em Genética.
Universidade Federal de Pernambuco, UFPE, Recife, Brasil
Título: Indução de mutação e seleção em feijão-caupi [Vigna unguiculata (L.) Walp.] visando tolerância à salinidade., Ano de obtenção: 2007

Orientador: Ederson Akio Kido
Bolsista do(a): Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco
*Palavras-chave: Indução de mutação, Salinidade, Vigna unguiculata, Radiação Gama, In vitro, Cultura de tecido*
*Áreas do conhecimento : Genética Vegetal,Fisiologia Vegetal,Mutagenese*
*Setores de atividade : Produção Vegetal*

**1999 - 2004** Graduação em Bacharelado em Ciencias Biologicas.
Centro de Ciências Biológicas, UFPE, Recife, Brasil
Título: Conhecimento e Uso de Plantas Medicinais por Especialistas Locais em um Fragmento de Mata Atlântica no Estado de Pernambuco
Orientador: Ulysses Paulino de Albuquerque
*Áreas do conhecimento : Ciências Biológicas*

### Formação complementar

**2006 - 2006** Curso de curta duração em Marcadores Moleculares no Melhoramento de Plantas. (Carga horária: 10h).
Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA, Brasília, Brasil

**2005 - 2005** Curso de curta duração em Téc Moleculares Bioinformática e Mapeamento Aplica. (Carga horária: 30h).
Centro Brasil Argentina de Biotecnologia, CBAB, Brasil

**2003 - 2003** Curso de curta duração em Aqüicultura. (Carga horária: 16h).
Confederação da Agricultura e Pecuária do Brasil, CNA, Brasil

**2002 - 2002** Curso de curta duração em Ccls Basic And Abbreviated Teaching Techniques. (Carga horária: 24h).
Centro Cultural Anglo Americano, C.C.A.A., Brasil

**2002 - 2002** Curso de curta duração em Abordagem Multidisciplinar da Fitoterapia. (Carga horária: 8h).
Associação Farmacêutica de Pernambuco, AFP*, Brasil

**2001 - 2001** Curso de curta duração em Ciclo Avançado de Língua Inglesa. (Carga horária: 100h).
Cooperativa de Ensino de Línguas, COPEL*, Brasil

**2000 - 2000** Curso de curta duração em Introdução a Hematologia. (Carga horária: 10h).
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

**2000 - 2000** Curso de curta duração em Ecoturismo: Planejamento e Gestão. (Carga horária: 10h).
Universidade Federal da Paraíba, UFPB, Joao Pessoa, Brasil

**2000 - 2000** Curso de curta duração em Introdução à Biologia Molecular. (Carga horária: 80h).
Universidade Federal de Pernambuco, UFPE, Recife, Brasil

**1999 - 1999** Curso de curta duração em Curso de Orquidofilia. (Carga horária: 20h).
Sociedade Orquidófila de Pernambuco, SOPE, Brasil

## Atuação profissional

### 1. Faculdade Maurício de Nassau - Recife - UNINASSAU

**Vínculo institucional**

**2009 - 2010** Vínculo: Prof. Microbiol. e Imunologia , Enquadramento funcional: Prof. Microbiol. e Imunologia , Carga horária: 8, Regime: Parcial
Outras informações:
Professor de Micobiologia e imunologia para o curso de Enfermagem da Maurício de Nassau-PE

**2008 - 2008** Vínculo: Prof. de Genética e Evolucão , Enquadramento funcional: Prof. de Genética e Evolucão , Carga horária: 20, Regime: Parcial
Outras informações:
Professor da Disciplina genética e Evolução para os cursos de Nutrição e Enfermagem da faculdade Mauricio de Nassau-PE

**2008 - 2010** Vínculo: Professor de Genética Humana , Enquadramento funcional: Professor de Genética Humana , Carga horária: 25, Regime: Parcial
Outras informações:
Professor de Genética Humana nos cusrsos de Enfermagem, Fisioterapia e Farmácia da Faculdade Maurício de Nassau - PE

**2008 - 2008** Vínculo: Prof. Bioq. e Biol. Molecular , Enquadramento funcional: Prof. Bioq. e Biol. Molecular , Carga horária: 6, Regime: Parcial
Outras informações:
Professor de Bioquímica e Biologia Molecular para o curso de Farmácia da faculdade Maurício de Nassau-PE

**Atividades**

**03/2010 - 07/2010** Graduação, Bacharelado em Fisioterapia

*Disciplinas ministradas:*
*Genética Humana*

**02/2008 - 07/2009** Graduação, Bacharelado em Nutrição

*Disciplinas ministradas:*
*Genética Humana , Genética e Evolução*

**02/2008 - 07/2010** Graduação, Bacharelado em Enfermagem

*Disciplinas ministradas:*
*Genética e Evolução , Genética Humana , Microbiologia e Imunologia*

**02/2008 - 07/2010** Graduação, Bacharelado em Farmácia

*Disciplinas ministradas:*
*Genética Humana , Bioquímica e Biologia Molecular*

### 2. Centro Cultural Anglo Americano - C.C.A.A.

**Vínculo institucional**

**2004 - 2004** Vínculo: Contratado , Enquadramento funcional: Professor , Carga horária: 16, Regime: Parcial
Outras informações:
Atuando como professor de Lingua Inglesa para Turma de nível básico a avançado.

**2004 - 2004** Vínculo: Contratado , Enquadramento funcional: Professor , Carga horária: 16, Regime: Parcial
Outras informações:
Atuando como professor de Lingua Inglesa para Turma de nível básico a avançado.

**2003 - 2003** Vínculo: Contratado , Enquadramento funcional: Professor , Carga horária: 16, Regime: Parcial
Outras informações:
Atuando como professor de Lingua Inglesa para Turma de nível básico a avançado.

**2002 - 2002** Vínculo: Contratado , Enquadramento funcional: Professor , Carga horária: 16, Regime: Parcial
Outras informações:
Atuando como professor de Lingua Inglesa para Turma de nível básico a avançado.

**Atividades**

**08/2002 - 08/2004** Aperfeiçoamento

*Especificação:*
*Língua Inglesa - ciclo básico , Língua Inglesa - ciclo intermediário , Língua Inglesa - ciclo avançado*

**3. Universidade Federal de Pernambuco - UFPE**

**Vínculo institucional**

**2005 - 2007** Vínculo: Mestrando , Enquadramento funcional: Pós-Graduando , Carga horária: 40, Regime: Integral
Outras informações:
Mestrando do Programa de Pós-graduação em Genética

**2002 - 2003** Vínculo: Monitoria ( FACEPE/CNPq) , Enquadramento funcional: Monitor , Carga horária: 20, Regime: Parcial
Outras informações:
Monitor da disciplina "Genética Mendeliana" realizada na CCB-Centro de Ciências Biológicas, UFPE

**2001 - 2002** Vínculo: Bolsa (FACEPE/CNPQ) , Enquadramento funcional: Iniciação Científica , Carga horária: 20, Regime: Parcial
Outras informações:
Desenvolvendo o projeto de pesquisa intitulado: "Desenvolvimento Técnico na Produção de Laticínios".

**2000 - 2004** Vínculo: Estudante , Enquadramento funcional: Graduando , Carga horária: 20, Regime: Dedicação exclusiva

**2000 - 2001** Vínculo: Bolsa (FACEPE/CNPq) , Enquadramento funcional: Iniciação Científica , Carga horária: 20, Regime: Parcial
Outras informações:
Desenvolvendo o projeto de pesquisa intitulado: "Cinética Fermentativa em Frasco de Células Recombinantes de Kluyveromyces marxianus em Soro de Queijo".

**Atividades**

**10/2005 - 10/2005** Conselhos, Comissões e Consultoria, Cbab Centro Brasil Argentina de Biotecnologia

*Especificação:*
*Integrante da comissão organizadora do III Workshop Recife Megacity - Open Space Project. Evento de abrangência internacional (ênfase para Alemanha e Aústria), financiado pelo Bundesministerium für Bildung und Forshung - BMBF, com carga horária de 90*

**09/2005 - 09/2005** Conselhos, Comissões e Consultoria, Cbab Centro Brasil Argentina de Biotecnologia

*Especificação:*
*Integrante da comissão organizadora do curso "Maps Markers Genomics in Plant Breeding - Técnicas Moleculares, Bioinformática e Mapeamento Aplicados ao Melhoramentode Plantas". Curso de abrangência internacional com 10 dias de duração (tempo integral)*

**06/2002 - 03/2003** Graduação, Bacharelado em Ciencias Biologicas

*Disciplinas ministradas:*
*Monitor da disciplina "Genética Mendeliana", com carga horária de 480 horas.*

**08/2001 - 07/2002** Pesquisa e Desenvolvimento, Centro de Ciências Biológicas, Departamento de Genética

*Linhas de pesquisa:*
*Desenvolvimento técnico na Produção de Laticínios (Desenvolvido no LIKA-Laboratório de Imunopatologia Keizo Asami)*

**08/2000 - 07/2001** Pesquisa e Desenvolvimento, Centro de Ciências Biológicas, Departamento de Genética

*Linhas de pesquisa:*
*Cinética Fermentativa em Frasco de Células Recombinantes de Kluyveromyces marxianus em Soro de Queijo (Desenvolvido no LIKA-Laboratório de Imunopatologia Keizo Asami)*

**01/2000 - 12/2002** Estágio, Centro de Ciências Biológicas, Departamento de Genética

*Estágio:*
*Estágio no LIKA - Laboratório de Imunopatologia Keizo Asami, Setor de biologia Molecular (Carga Horária total: 2.160 horas)*

**Linhas de pesquisa**

1. Cinética Fermentativa em Frasco de Células Recombinantes de Kluyveromyces marxianus em Soro de Queijo (Desenvolvido no LIKA-Laboratório de Imunopatologia Keizo Asami)
2. Desenvolvimento técnico na Produção de Laticínios (Desenvolvido no LIKA-Laboratório de Imunopatologia Keizo Asami)

## Áreas de atuação

1. Genética Vegetal
2. Cultura de Tecido Vegetal
3. Mutagenese
4. Genética Molecular e de Microorganismos
5. Expressão Heteróloga
6. Ensino Superior

## Idiomas

**Inglês** Compreende Bem , Fala Bem , Escreve Bem , Lê Bem

## Producão

### Produção bibliográfica

#### Artigos completos publicados em periódicos

1. **GAZZANEO, L. R. S.**, COLACO, W., KIDO, E. A., BENKO-ISEPPON, A. M., HOULLOU-KIDO, L. M.
Efeito da Radiação Gama Sobre o Desenvolvimento in vitro de Vigna unguiculata. Revista Brasileira de Biociências. , v.5, p.27 - 29, 2007.
*Palavras-chave: In vitro, Cowpea, Vigna unguiculata, Indução de mutação, Radiação Gama*
*Referências adicionais : Português. Meio de divulgação: Meio digital. Home page: [http://www6.ufrgs.br/seerbio/ojs/index.php/rbb/article/view/73/70]*

2. **GAZZANEO, L. R. S.**, COLACO, W., ALVES, G. D., KIDO, E. A., BENKO-ISEPPON, A. M., HOULLOU-KIDO, L. M.
Efeito da Radiação Gama Sobre o Desenvolvimento in vivo de Vigna unguiculata (L.) Walp.. Revista Brasileira de Biociências. , v.5, p.81 - 83, 2007.
*Referências adicionais : Português. Meio de divulgação: Meio digital. Home page: [http://www6.ufrgs.br/seerbio/ojs/index.php/rbb/article/view/115/111]*

3. **GAZZANEO, L. R. S.**, LUCENA, Reinaldo Farias Paiva de, ALBUQUERQUE, Ulysses Paulino de
Knowledge and use of medicinal plants by local specialists in an region of Atlantic Forest in the state of Pernambuco (Northeastern Brazil). Journal of Ethnobiology and Ethnomedicine. JCR, 2005.
*Palavras-chave: Atlantic Forest, Ethnobotany, Medicinal plant, Ethnomedicine*
*Áreas do conhecimento : Etnobotânica*
*Setores de atividade : Saúde Humana*
*Referências adicionais : Brasil/Inglês. Meio de divulgação: Hipertexto. Home page: http://www.ethnobiomed.com/content/1/1/9*

#### Artigos aceitos para publicação

1. **GAZZANEO, L. R. S.**, PANDOLFI, V., JESUS, A. L. S., CROVELLA, S., BENKO-ISEPPON, A. M., FREITAS, A. C.
Heterologous Expression Systems for Plant DefensinExpression: Examples of Success and Pitfalls. Current Protein and Peptide Science. JCR, 2016.
*Palavras-chave: E. coli, fusion, carrier protein, secretion signal, inclusion bodies, antimicrobial peptides*
*Áreas do conhecimento : Expressão Heteróloga,Biotecnologia,Genética Molecular e de Microorganismos*
*Referências adicionais : Inglês.*

2. HOULLOU-KIDO, L. M., **GAZZANEO, L. R. S.**, BENKO-ISEPPON, A. M., ALVES, G. D., KIDO, E. A.
Efeito da radiação gama sobre o desenvolvimento in vivo e in vitro de Vigna unguiculata (L.) Walp.. Pesquisa Agropecuária Pernambucana. , 2007.
*Palavras-chave: Vigna unguiculata, In vitro, Radiação Gama, Indução de mutação*
*Referências adicionais : Português.*

#### Trabalhos publicados em anais de eventos (resumo)

1. **GAZZANEO, L. R. S.**, HOULLOU-KIDO, L. M., KIDO, E. A.
INFLUÊNCIA DO ESTÁDIO FISIOLÓGICO NA REGENERAÇÃO IN VITRO DE VIGNA UNGUICULATA In: X Congresso Nacional de Fisiologia Vegetal / XII Congresso Latino-Americano de Fisiologia Vegetal, 2005, Recife.
**Brazilian Journal of Plant Physiology**. Brazilian Society of Plant Physiology, 2005. v.17. p.465 - 465
*Palavras-chave: Vigna unguiculata, Caupi, In vitro, Regeneração*
*Áreas do conhecimento : Biotecnologia,Cultura de Tecido Vegetal,Melhoramento Vegetal*
*Referências adicionais : Brasil/Português. Meio de divulgação: Meio digital*

2. **GAZZANEO, L. R. S.**
Utilização da Enzima ß-Glicosidase como Marcador Seletivo no Processo de Transformação Genética de Linhagens Industriais de Saccharomyces cerevisiae In: 6 Jornada de Iniciação Científica - ITEP 60 Anos, 2002, Recife.
. , 2002.
*Palavras-chave: ß-glicosidade, Saccharomyces, Recombinante, Fermentação, Álcool*
*Áreas do conhecimento : Genética Molecular e de Microorganismos*
*Setores de atividade : Produção de Álcool*
*Referências adicionais : Brasil/Português. Meio de divulgação: Impresso*

3. **GAZZANEO, L. R. S.**
Cinética fermentativa em frascos de células recombinantes de Kluyveromyces marxianus em soro de queijo In: V Jornada de Iniciação Científica, 2001, Recife.
. , 2001.
*Palavras-chave: Fermentação, kluyveromyces, soro de queijo*
*Áreas do conhecimento : Biotecnologia*
*Setores de atividade : Produtos e Processos Biotecnológicos Vinculados À Agricultura*
*Referências adicionais : Brasil/Português. Meio de divulgação: Impresso*

#### Trabalhos publicados em anais de eventos (resumo expandido)

1. SILVA, Mário Correia da, PINANGÉ, Diego Sousa Barros, CRUZ, Geyner Alves S, BORTOLETI, Kyria Cilene A, BERNARDES, Ebenezer C S, AZEVEDO, Hayana M A, **GAZZANEO, L. R. S.**, HOULLOU-KIDO, L. M., KIDO, E. A., BENKO-ISEPPON, A. M.
Avaliação cromossômica convencional e de bandeamento em indivíduos de feijão-caupi germinados e regenerados in vitro In: Congresso Nacional de Feijão-Caupi / VI Reunião Nacional de Feijão-Caupi, 2006, Teresina-PI.
**Congresso Nacional de Feijão-Caupi / VI Reunião Nacional de Feijão-Caupi**. , 2006.
*Palavras-chave: Citogenética, Caupi, Bandeamento, In vitro, cromossomos*
*Áreas do conhecimento : Citogenética*
*Setores de atividade : Outros*
*Referências adicionais : Brasil/Português. Meio de divulgação: Impresso*

2. HOULLOU-KIDO, L. M., **GAZZANEO, L. R. S.**, MELO, A. P., BENKO-ISEPPON, A. M., KIDO, E. A.
Influência da Contaminação Endofítica no Potencial Regenerativo invitro do Feijão-Caupi In: Congresso Nacional do Feijão-Caupi / VI Reunião Nacional de Feijão-Caupi, 2006, Teresina.
**Congresso Nacional do Feijão-Caupi / VI Reunião Nacional de Feijão-Caupi**. , 2006.
*Palavras-chave: Vigna unguiculata, In vitro, Contaminação, Fungo*
*Referências adicionais : Brasil/Português. Meio de divulgação: Meio digital*

#### Apresentação de trabalho e palestra

1. SANTANA, K. C. B., AMORIM, L. L. B., PANDOLFI, V., BELARMINO, L. C., **GAZZANEO, L. R. S.**, KIDO, E. A., CROVELLA, S., BENKO-ISEPPON, A. M.
**Caracterização defensina putativa e isolamento de seu gene codificante em Euphorbia hyssopifolia L.: Novas perspectivas terapêuticas.**, 2011. (Congresso,Apresentação de Trabalho)
*Palavras-chave: peptídeos antimicrobianos, GENÉTICA VEGETAL, proteínas medicinais*
*Áreas do conhecimento : Genética Vegetal,Genética Molecular e de Microorganismos,Farmacologia*
*Referências adicionais : Brasil/Português. Meio de divulgação: Impresso; Local: Mar Hotel; Cidade: Recife; Evento: 2 Encontro Brasileiro de Inovação Terapêutica; Inst.promotora/financiadora: Programa de Pós-Graduação em Inovação Terapêutica (UFPE)*

2. BELARMINO, L. C., CRUZ, H. L. A., **GAZZANEO, L. R. S.**, SANTANA, K. C. B., PANDOLFI, V., CROVELLA, S., ABDELNOOR, R., BENKO-ISEPPON, A. M.
**Orchestration of the response to enviromental stress: defensins play their part.**, 2011. (Congresso,Apresentação de Trabalho)
*Palavras-chave: defensina, bioinformática, fisiologia vegetal*
*Áreas do conhecimento : Genética Vegetal,Bioinformática*
*Referências adicionais : Brasil/Inglês. Meio de divulgação: Impresso; Local: São Paulo; Cidade: Águas de Lindóia; Evento: 57 Congresso Brasileiro de Genética; Inst.promotora/financiadora: SBG - Sociedade Brasileira de Genética*

3. AMORIM, L. L. B., SANTANA, K. C. B., **GAZZANEO, L. R. S.**, PANDOLFI, V., BELARMINO, L. C., BEZERRA-NETO, J. P., CROVELLA, S., KIDO, E. A., BENKO-ISEPPON, A. M.
**Sequence analysis and homology modeling of the first defenin from Etlingera elatior**, 2011. (Conferência ou palestra,Apresentação de Trabalho)
*Palavras-chave: modelagem protéica, bioinformática, defensina*
*Áreas do conhecimento : Bioinformática,Genética Vegetal*
*Referências adicionais : Brasil/Inglês. Meio de divulgação: Impresso; Local: Santa Catarina - SC; Cidade: Florianópolis; Evento: X-Meeting 2011 (AB3C / SolBio) / 7th International Conference os the Brazilian Association for Bioinformatics and Computational Biology / 3rd international Conference of the liberoAmerican Society of Bioinformatics; Inst.promotora/financiadora: AB3C / SolBio*

4. **GAZZANEO, L. R. S.**, KIDO, E. A., HOULLOU-KIDO, L. M., BENKO-ISEPPON, A. M.
**Efeito da Radiação Gama Sobre o Desenvolvimento in vitro de Vigna unguiculata (L.) Walp.**, 2006. (Congresso,Apresentação de Trabalho)
*Palavras-chave: Caupi, Vigna unguiculata, Cowpea, Indução de mutação, Radiação Gama*
*Áreas do conhecimento : Biotecnologia,Cultura de Tecido Vegetal,Melhoramento Vegetal*
*Referências adicionais : Brasil/Português. Meio de divulgação: Impresso; Local: FAURGS - UFRGS; Cidade: Gramado; Inst.promotora/financiadora: 57 Congresso Brasileiro de Botânica*

5. **GAZZANEO, L. R. S.**, KIDO, E. A., HOULLOU-KIDO, L. M., BENKO-ISEPPON, A. M.
**Efeito da Radiação Gama Sobre o Desenvolvimento in vivo de Vigna unguiculata (L.) Walp.**, 2006. (Congresso,Apresentação de Trabalho)
*Referências adicionais : Brasil/Português. Meio de divulgação: Impresso; Local: FAURGS - UFRGS; Cidade: Gramado; Inst.promotora/financiadora: 57 Congresso Brasileiro de Botânica*

## Orientações e Supervisões

**Orientações e supervisões**

### Orientações e supervisões concluídas

### Trabalhos de conclusão de curso de graduação

1. Manuela Barbosa Negreiros e Silvana Lúcia L. V. Melo. **A inferilidade como conseqüência da endometriose**. 2010. Curso (Bacharelado em Enfermagem) - Faculdade Maurício de Nassau - Recife
*Palavras-chave: agonistas, endométrio, refluxo transtubário*
*Áreas do conhecimento : Saúde Materno-Infantil*
*Setores de atividade : Atividades de atenção à saúde humana*
*Referências adicionais : Brasil/Português.*

## Eventos

**Eventos**

### Participação em eventos

1. **Ciclo de Palestras em Genética e Biologia Molecular**, 2007. (Seminário)
.

2. Apresentação de Poster / Painel no(a) **Congresso Nacional do Feijão-Caupi / VI Reunião Nacional de Feijão-Caupi**, 2006. (Congresso)
Congresso Nacional do Feijão-Caupi / VI Reunião Nacional de Feijão-Caupi.
*Palavras-chave: Feijão-Caupi, Caupi, Cowpea, Vigna unguiculata*
*Áreas do conhecimento : Melhoramento Vegetal*
*Setores de atividade : Produtos e Processos Biotecnológicos Vinculados Â Agricultura*

3. Apresentação de Poster / Painel no(a) **57º Congresso Nacional de Botânica**, 2006. (Congresso)
Efeito da Radiação Gama Sobre o Desenvolvimento in vivo de Vigna unguiculata (L.) Walp..

4. **XI Agrinordeste - Seminários sobre a Modernização do Setor Primário a Economia Nordestina**, 2003. (Seminário)
.
*Palavras-chave: Seminário*
*Áreas do conhecimento : Ciências Agrárias*
*Setores de atividade : Agricultura, Pecuária, Silvicultura, Exploração Florestal*

5. **I Jornada Pernambucana de Plantas Medicinais e Fitoterapia**, 2002. (Congresso)
I Jornada Pernambucana de Plantas Medicinais e Fitoterapia.
*Palavras-chave: Congresso*
*Áreas do conhecimento : Etnofarmacologia*

6. Apresentação de Poster / Painel no(a) **VI Jornada de Iniciação Científica**, 2002. (Encontro)
VI Jornada de Iniciação Científica.
*Palavras-chave: iniciação científica, Bolsista*
*Áreas do conhecimento : Biotecnologia na Produção de Álcool*
*Setores de atividade : Produção de Álcool*

7. **Encontro Internacional - Eficiência nda Comercialização**, 2002. (Encontro)
.
*Palavras-chave: Congresso*
*Áreas do conhecimento : Mercadologia*
*Setores de atividade : Logística de Transporte, Armazenagem e Comunicações*

8. Apresentação de Poster / Painel no(a) **V Jornada de Iniciação Científica**, 2001. (Encontro)
V Jornada de Iniciação Científica.
*Palavras-chave: iniciação científica, Bolsista*
*Áreas do conhecimento : Biotecnologia*
*Setores de atividade : Produtos e Processos Biotecnológicos*

9. **I Semana de biologia Animal da UFPE**, 2001. (Encontro)
.
*Palavras-chave: Encontro*

*Areas do conhecimento : Zoologia*

10. **I Encontro Nordestino de Biogeografia -**, 2000. (Encontro)
.
*Palavras-chave: Encontro*
*Áreas do conhecimento : Ecologia de Ecossistemas*

11. **Seminário de Atualização em Fisiologia - A Comunicação Celular**, 1999. (Seminário)
Atualização em Fisiologia - A Comunicação Celular.
*Palavras-chave: Seminário*
*Áreas do conhecimento : Fisiologia Celular*

12. **II Encontro Interno dos Estudantes de Biologia - Cidadão Biólogo**, 1999. (Encontro)
.
*Áreas do conhecimento : Ciências Biológicas*

13. **II Oficina de Identificação Botânica - Subclasse Rosidae**, 1999. (Oficina)
.
*Palavras-chave: Oficina*
*Áreas do conhecimento : Taxonomia de Fanerógamos*

### Organização de evento

1. **GAZZANEO, L. R. S.**, BENKO-ISEPPON, A. M., KIDO, E. A.
**Curso Genes, Genomas e Tecnologias Genômicas**, 2006. (Outro, Organização de evento)
*Referências adicionais : Brasil/Português. Meio de divulgação: Vários*

2. **GAZZANEO, L. R. S.**, BENKO-ISEPPON, A. M., KIDO, E. A.
**Workshop Recife Megacity: Open Space Project**, 2005. (Outro, Organização de evento)
*Referências adicionais : Brasil/Inglês. Meio de divulgação: Outro*

## Bancas

**Bancas**

### Participação em banca de trabalhos de conclusão

### Graduação

1. Batista, J. M. S., Gomes, A. M. A., Rodrigues, V. J. L. B., **GAZZANEO, L. R. S.**
Participação em banca de Joyce Maria dos Santos Batista. **Levantamento de plantas medicinais comercializadas em mercados públicos de recife e Olinda - PE e utilizadas como antiinflamatórios**, 2010
(Bacharelado em Farmácia) Faculdade Maurício de Nassau - Recife
*Palavras-chave: Etnobotânica, plantas medicinais, antiinflamatórios*
*Áreas do conhecimento : Etnobotânica,Farmácia*
*Setores de atividade : Atividades de atenção à saúde humana*
*Referências adicionais : Brasil/Português.*

2. Paloma Rafalella de Silva Araújo, Elizangela Ramos Castanha, **GAZZANEO, L. R. S.**
Participação em banca de Paloma Rafaella de Silva Araújo. **Pseudomonas aeruginosa: A incidência de cepas multiresistentes em infecções hospitalares e seus mecanismos de resistência.**, 2010
(Bachalrelado em Biomedicina) Faculdade Maurício de Nassau - Recife
*Palavras-chave: Infecções, Multiresistência, Pseudomonas*
*Áreas do conhecimento : Microbiologia*
*Setores de atividade : Atividades de atenção à saúde humana*
*Referências adicionais : Brasil/Português.*

3. Gonçalves, V. S., Maia, W. B., Neto, J. H. R. C., **GAZZANEO, L. R. S.**
Participação em banca de Vanessa de Souza Gonçalves. **Uso de medicamentos teratogênicos durante a gestação com ênfase no uso contínuo de anticonvulsivantes.**, 2010
(Bacharelado em Farmácia) Faculdade Maurício de Nassau - Recife
*Palavras-chave: teratogenos, anticonvulsivantes*
*Áreas do conhecimento : Farmácia*
*Setores de atividade : Atividades de atenção à saúde humana*
*Referências adicionais : Brasil/Português.*

4. Filho, G. J. F. C., AZEVEDO, Hayana M A, BENKO-ISEPPON, A. M., **GAZZANEO, L. R. S.**
Participação em banca de Giovani José Feitosa Cavalcanti Filho. **Estabelecimento de protocolo de mutagênese in vitro para Celosia argentea L.**, 2009
(Bacharelado em Ciencias Biologicas) Universidade Federal de Pernambuco
*Palavras-chave: mutagênese, In vitro, celosia*
*Áreas do conhecimento : Melhoramento Vegetal,Mutagenese*
*Setores de atividade : Agricultura, Pecuária, Produção Florestal, Pesca e Aqüicultura*
*Referências adicionais : Brasil/Português.*

## Totais de produção

**Produção bibliográfica**

| | |
|---|---|
| Artigos completos publicados em periódico | 3 |
| Artigos aceitos para publicação | 2 |
| Trabalhos publicados em anais de eventos | 5 |
| Apresentações de trabalhos (Conferência ou palestra) | 1 |
| Apresentações de trabalhos (Congresso) | 4 |

**Orientações**

| | |
|---|---|
| Orientação concluída (trabalho de conclusão de curso de graduação) | 1 |

**Eventos**

| | |
|---|---|
| Participações em eventos (congresso) | 3 |
| Participações em eventos (seminário) | 3 |

| | |
|---|---|
| Participações em eventos (oficina) | 1 |
| Participações em eventos (encontro) | 6 |
| Organização de evento (outro) | 2 |
| Participação em banca de trabalhos de conclusão (graduação) | 4 |

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