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Aplicações biotecnológicas em feijão-caupi [*Vigna unguiculata* (L.) Walp.] visando a obtenção de tolerância a estresses bióticos e abióticos.

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Tese apresentada ao curso de Doutorado do Programa de Pós Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos obrigatórios para obtenção do título de Doutor em Ciências Biológicas, na área de concentração Biotecnologia/Biologia Celular e Molecular.

Orientador: Profª Drª Ana Maria Benko-Iseppon
Co-orientador: Francisco José Lima Aragão

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Morte não existe. Tudo é quântico!
Em cada acontecimento feliz você estará aqui,
sem nem que eu perceba essa distância entre corpo e alma.
Choro sim, mas com uma grande certeza
de que o vazio vai dar espaço à celebração,
porque foi assim que você me ensinou. Amar e celebrar.
Te amo incondicionalmente,
Hayana Azevedo.
Recife, 25 de outubro de 2012.

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“Sempre permaneça aventureiro.
Por nenhum momento se esqueça de que
a vida pertence aos que investigam.
Ela não pertence ao estático;
Ela pertence ao que flui.
Nunca se torne um reservatório,
sempre permaneça um rio.”

“A menos que você aceite com gratidão tudo o que a vida traz,
você está deixando escapar o sentido.”

Osho

Resumo

O feijão-caupi (*Vigna unguiculata*) vem aumentando gradativamente seu potencial produtivo. Contudo, a área colhida, a produção e a produtividade oscilam muito em virtude das variações climáticas, bem como os ataques de pragas e patógenos. Estudos moleculares aliados a ferramentas biotecnológicas, como a transgenia e a mutagênese, podem contribuir na redução dos problemas gerados pelos estresses. O presente trabalho teve como objetivo inserir um gene ligado à defesa na cultivar BRS Tumucumaque de feijão-caupi, conferindo-lhe maior tolerância a estresses bióticos. Para obtenção dos resultados, foi utilizada a metodologia de transferência direta de genes via Biobalística. Foram bombardeados cerca de 700 embriões e cinco exemplares (0,7%) passaram por todas as etapas da pressão de seleção, chegando à aclimatação. Uma vez aclimatadas, realizou-se a análise por PCR. Para confirmação da transformação foi feito uso do conjunto de *primers* pAHAS 124/pAHAS 500. Os *primers*foram utilizados para amplificar uma sequência de 624 pb e, em conjunto com a sequência codificante, cerca de 750 pb. Em um segundo momento, também objetivou-se gerar mutantes com características de tolerância a estresse salino, fazendo uso da mutagênese *in vitro* através de irradiação por raios gama sobre o desenvolvimento *in vitro* da cv. BR14-Mulato.Inicialmente, foi estabelecido um protocolo apropriado para pressão de seleção *in vitro* das sementes irradiadas, fazendo-se uso das concentrações de 172 mM, 258 mM, 344 mM e 0 mM, chegando-se, então, à conclusão de que a concentração de 172 mM seria a mais apropriada. Por fim, sementes foram irradiadas com três doses de radiação gama (100 Gy, 150 Gy e 200 Gy), além do material controle (0 Gy), e seus tecidos foram isolados e inoculados, simultaneamente, em meios de cultura com e sem sal. Como resultado, foi obtido um possível variante somaclonal advindo de material não irradiado e inoculado em meio não seletivo, sendo transferido, posteriormente, para meio contendo NaCl, sobrevivendo a este. Outro resultado de grande importância foi a obtenção de um provável mutante sólido provocado pela dose de 150 Gy que, inicialmente, formou calo embriogênico na ausência de NaCl, sendo posto sob pressão de seleção na etapa conseguinte.

Palavras-chave: Transgênico. Feijão-caupi. Biobalística. Engenharia genética. Defensina. Peptídeos antimicrobianos.Mutagênese.

Abstract

Cowpea (*Vigna unguiculata*) is gradually increasing its productive potential. However, the harvested area, production and productivity of this crop oscillate due to climate changes, as well as attacks by pests and pathogens. Molecular studies aiming the isolation of genes combined with biotechnological tools, such as transgenesis and mutagenesis, can contribute effectively in reducing the problems caused by stress. The present work aimed to insert a defense gene in thecowpea cultivar BRS Tumucumaque,conferring increased tolerance to biotic stresses. To obtain these results, we used the methodology of direct gene transfer via biolistic. About 700 embryos were bombarded and five samples (0.7%) went through all the stages of selection pressure, reaching acclimation. Once acclimated, they were analyzed by PCR. For confirmation of the transformation a set of primers (AHAS124/pAHAS500) were used. In conjunction with the coding sequence, the pair showed a band of approximately 750 bp.In a second moment, was also aimed to generate mutants with characteristics of salt stress tolerance, making use of in vitro mutagenesis through gamma irradiation on the development in vitro of the cv. BR14-Mulato, from determining the minimum concentration of sodium chloride (NaCl) capable of inhibiting in vitro regeneration of tissues and subsequent choice of the radiation level capable of generating genetic variation.Initially, was established an appropriate protocol for selection pressure of the irradiated seeds, making use of concentrations of 172 mM, 258 mM, 344 mM and 0 mM, coming then to the conclusion that the concentration of 172 mM would be more appropriate. Finally, seeds were irradiated with three doses of gamma radiation (100 Gy, 150 Gy and 200 Gy), and their tissues were isolated and inoculated simultaneously in culture media with and without salt. As a result, were obtained a possible somaclonal variation arising from non-irradiated materials and inoculated onto non-selective medium, being transferred later to medium containing NaCl, surviving this. Another was the achievementof a mutant caused by the dose of 150 Gy, which originally formed embryogenic callus in the absence of NaCl and later placed under selection pressure in the next step.

Keywords: Transgenic. Cowpea. Biolistic. Genetic engineering. Defensin. Antimicrobial peptides. Mutagenesis.

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Lista de Siglas Abreviaturas

AMP	*Antimicrobial Peptides*, Peptídeos Antimicrobianos
CaMV	*Cauliflower Mosaic Virus*, Vírus do Mosaico da Couve-Flor
Cenargen	Centro Nacional de Recursos Genéticos e Biotecnologia
CNPAF	Centro Nacional de Pesquisa de Arroz e Feijão
CTAB	*Cetyl-trimethyl-amoniumbromide*
Cv	Cultivar
DNA	*Desoxyribonucleic Acid*; Ácido Desoxirribonucleico
Embrapa	Empresa Brasileira de Pesquisa Agropecuária
FACEPE	Fundação de Amparo a Ciência e Tecnologia do Estado de Pernambuco
Kg	Quilograma
Ha	Hectare
IPA	Instituto Agronômico de Pernambuco
LB	Luria Bertani
MCS	*Multiple Cloning Site*, Sítio de Clonagem Múltipla
Mg	Miligrama
mL	Mililitro
µL	Microlitro
mM	Milimolar
Ng	Nanograma
Nos	Nopalina sintase
PR	*Pathogene Related*, Relacionado à Patogênese
PRR	*Pattern Recognition Receptors*, Receptores de Reconhecimento de Padrão
p.s.i.	*Pound Force Per Square Inch*, Libra Força Por Polegada Quadrada
Rpm	Rotação por minuto
SAR	*Sistemic Acquired Resistance*, Resistência Sistêmica Adquirida
T	Tonelada

Sumário

1 Introdução

O feijão-caupi [*Vigna unguiculata* (L.) Walp.]apresenta grande importância para a subsistência de milhões de pessoas relativamente pobres em países menos desenvolvidos dos trópicos(QUIN, 1997).No Brasil, em virtude de seu sistema de produção exigir muito trabalho manual, principalmente na colheita e na debulha, seu cultivo predomina na agricultura familiar nas regiões Norte e Nordeste, além disso, faltam tecnologias ao pequeno agricultor (FREIRE-FILHO *et al.*, 2011) tanto na forma de produzir quanto na forma de minimizar os danos gerados pelos estresses bióticos e abióticos.

De acordo com Freire-Filho *et al.* (2011), ainda no Brasil, a participação do feijão-caupi na região Norte, tanto na área cultivada quanto na produção é pequena; entretanto, a produtividade é praticamente igual à média nacional. A região Nordeste tem uma grande participação na área cultivada, porém apresenta uma parcela relativamente modesta da produção, a qual decorre da baixa produtividade.

Estudos moleculares voltados ao isolamento de genes,especialmente aliados ao uso de ferramentas biotecnológicas como a transgenia e a mutagênese,têm potencial de contribuir eficientemente na redução dos problemas gerados pelas adversidades. Considera-se que a intervenção da transformação vegetal pode facilitar a seleção e o isolamento de linhagens tolerantes (EL-SAYED *et al.*, 2007), havendo estratégias bem sucedidas na incorporação de níveis de tolerância em diferentes espécies (NOVAK e BRUNNER, 1992). Estudos desse tipo em leguminosas como, por exemplo, soja e feijão-caupi auxiliam na introdução das características de interesse em outras leguminosas, justificando estudos de genômica funcional e aplicações da engenharia genética na introdução de características desejáveis para diferentes cultivares de feijão-caupi.

Outra aplicação biotecnológica de grande demanda é a ‘mutagênese *in vitro*’. Essa técnica tem resultado em incrementos da variabilidade genética das espécies, uma vez que utiliza doses muito baixas de radiação, as quais normalmente causam apenas mutações pontuais (substituições, inserções ou deleções). Sem gerar grandes alterações no genoma. A viabilidade da técnica é comprovada por dados fornecidos pela FAO/IAEA[1] (*Food and Agriculture Organization, International Atomic*

[1]Disponível em: <http://mvgs.iaea.org>. Acesso em: 18 Jun. 2013.

Energy Agency) onde atualmente estão registradas aproximadamente 3200 variedades obtidas por mutação.

As plantas são organismos constantemente expostos a vários fatores adversos, contudo, possuem a capacidade natural de regular uma rede de vias de sinalização com respostas rápidas e eficazes desencadeadas por estresses bióticos e abióticos(PENNINCKX *et al.*, 1996).Entretanto, devido à relação coevolutiva interespecífica entre plantas e agentes patogênicos, as respostas de defesa aos ataques bióticos são extremamente variáveis(BILGIN *et al.*, 2010).

Apesar dos ataques de vários tipos de patógenos, o estabelecimento de doença em um indivíduo é exceção, o que mostra que todo organismo vivo, seja ele animal, planta ou microrganismo, deve ter desenvolvido mecanismos de defesa intrínsecos eficientes contra ataques desse tipo.O mais comum dos mecanismos faz uso da imunidade inata que envolve uma estratégia de defesa a partir da produção de peptídeos antimicrobianos como as defensinas (AERTS *et al.*, 2008). Plantas superiores possuem complexos e eficientes mecanismos de defesa contra diversos patógenos que levam à ativação transcricional de uma cascata de genes de diferentes vias metabólicas, incluindo aquelas responsáveis pela produção de defensinas (CHEN *et al.*, 2008; BENKO-ISEPPON *et al.*, 2010).

Para se desvendar o modo de ação das defensinas vegetais e identificar seus vários alvos, é crucial que se obtenham proteínas de defensinas puras e nativas em quantidade suficiente para a condução de estudos funcionais. Além disso, a produção de defensinas de plantas recombinantes idênticas às formas nativas é um passo essencial para se estudar as inúmeras atividades biológicas e os alvos dessas proteínas(MARQUES *et al.*, 2009).

O presente estudo teve como primeiro objetivo inserir um gene codificante, a partir de técnica de transgenia via biobalística, de defensina vegetal em uma cultivar produtiva e bem adaptada de feijão-caupi, de modo a conferir-lhe maior tolerância a patógenos em geral, especialmente considerando-se as condições de cultivo no nordeste brasileiro.

Em um segundo momento, também objetivou-se gerar mutantes com características de tolerância a estresse salino, fazendo uso da mutagênese *in vitro* através de irradiação por raios gama sobre o desenvolvimento *in vitro* da cv. BR14-Mulato, a partir da determinação da concentração mínima de cloreto de sódio (NaCl)

capaz de inibir a regeneração *in vitro* dos tecidos e posterior escolha do nível de radiação capaz de gerar variabilidade genética.

2 Objetivos

2.1 Geral

Otimizar protocolos de transformação genética via biobalística e mutagênese de cultivar de feijão-caupi tolerante a estresses bióticos e abióticos inerentes ao cultivo no nordeste brasileiro através do estabelecimento de protocolo de transformação genética via biobalística com herança estável das características pretendidas e da mutagênese *in vitro*.

2.2 Específicos

- Adaptar protocolo de transformação genética por biobalística para a cultivar selecionada (determinação da distância e da pressão de disparo mais adequada à introdução do gene alvo).

- Caracterizar por técnicas moleculares possíveis transgênicos selecionados *in vitro*.

- Estabelecer a concentração mínima de NaCl capaz de inibir o desenvolvimento das sementes para, assim, determinar os níveis de pressão de seleção.

- Induzir a mutaçãoo *in vitro* por radiação fama, com uma avaliaçãoo pr´via dos níveis de radiação eficientes para este fim.

- Acompanhar o processo de regeneração de plantas induzidas e não induzidas sob as condições de estresse salino *in vitro* pré-determinadas.

3.Revisão bibliográfica

3.1 Feijão-caupi

Importante leguminosa utilizada na alimentação humana, no Brasil, o feijão-caupi (*Vigna unguiculata*L. Walp.) vem aumentando gradativamente seu potencial produtivo ao longo dos anos, com produção concentrada nas regiões Nordeste e Norte, mas se expandindo para a região Centro-Oeste (Figura 1), principalmente para o estado do Mato Grosso (FREIRE-FILHO *et al.*, 2011).

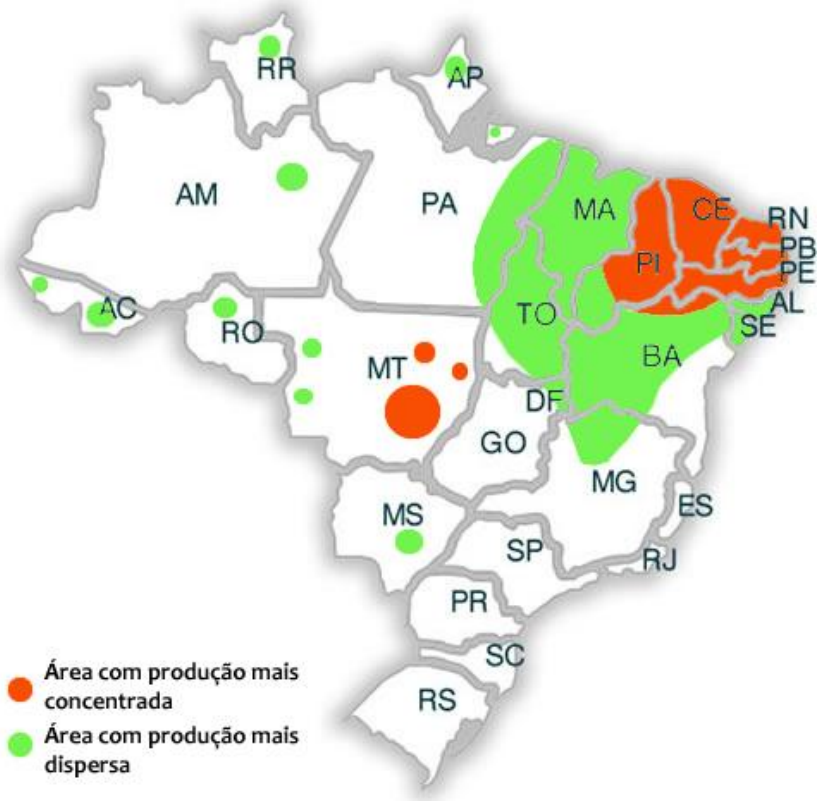

Figura 1 – Distribuição aproximada das regiões produtoras de feijão-caupi no Brasil. Adaptado de FREIRE-FILHO *et al.* (2011).

De acordo com a Embrapa Arroz e Feijão – CNPAF (2012), no Brasil, a média anual da área colhida do feijão-caupi, em 2011, foi de 1.687.304 ha, a produção de 782.966 t e a produtividade de 464 kg/ha. Com base nesses dados e considerando que cada hectare gera, em média, 0,8 emprego/ano e que o consumo per capita é de 18,21 kg/pessoa/ano, estima-se que no ano em questão a cultura tenha gerado 1,3 milhões de empregos, produzindo suprimento alimentar para aproximadamente

43 milhões de pessoas. Ainda segundo a Embrapa CNPAF (2012), em 2011 o feijão-caupi teve o maior rendimento dos últimos 25 anos (Tabela 1).

Apesar de a região Nordeste do Brasil apresentar a maior área de produção de feijão-caupi, sua produtividade é muito menor do que a esperada. Dessa forma, observa-se que apenas o Mato Grosso teve, em 2011, um rendimento de 963 kg/ha, enquanto que o Nordeste apresentou menos da metade do rendimento desse estado (EMBRAPA ARROZ E FEIJÃO – CNPAF, 2012).

De acordo com Freire-Filho *et al.*(2011), as informações citadas anteriormente justificam-se devido ao fato de a produção de feijão-caupi nas regiões Nordeste e Norte ser feita por empresários e agricultores familiares que ainda utilizam práticas tradicionais. Os autores continuam relatando que na região Centro-Oeste, onde o feijão-caupi passou a ser cultivado em larga escala a partir de 2006, a produção provém principalmente de médios e grandes empresários que praticam uma lavoura altamente tecnificada.Portanto, na região Nordeste, particularmente, para aumentar a produção não é necessário aumentar a área cultivada, mas investir em tecnologias.

Visando minimizar os problemas que acometem a cultura, já foram estabelecidos diversos protocolos na área de engenharia genética com objetivos diferentes, mas todos tendo como base o desenvolvimento de exemplares tolerantes aos estresses inerentes às áreas de produção. Porém, apenas 10 anos após o desenvolvimento da primeira planta transgênica é que se produziu o primeiro feijão-caupi geneticamente modificado a partir da metodologia de transferência via *Agrobacterium tumefaciens*(MUTHUKUMAR *et al.*, 1996). Além disso, houve um tempo considerável para o aparecimento do primeiro trabalho de transformação da espécie usando a técnica de Biobalística (IKEA *et al.*, 2003).

No Brasil, Ivo *et al.*(2008) desenvolveram, por sua vez, o primeiro processo eficiente de transformação de feijão-caupi, apresentando elevada frequência de transformantes e estabilidade na herança da característica. A viabilidade dessa abordagem, assim como o uso do Imazapyr (molécula herbicida capaz de translocar-se sistematicamente em direção à região apical da planta), trouxe à tona o enorme potencial deste sistema de produção de cultivares expressando características de interesse agronômico (CITADIN *et al.*, 2011).

Tabela 1 – Dados de conjuntura da produção de feijão-caupi no Brasil (1985 – 2011). Adaptado de Embrapa Arroz e Feijão – CNPAF (2012).

Ano	Área (ha)	Produção (t)	Rendimento (kg/ha)
1985	588.256	146.458	249
1986	688.455	212.908	319
1987	545.207	99.730	183
1988	649.253	244.321	376
1989	612.778	193.878	316
1990	796.116	225.720	284
1991	1.765.254	619.238	351
1992	1.654.980	325.216	197
1993	780.906	196.052	251
1994	1.989.025	759.372	382
1995	1.666.960	556.104	334
1996	1.581.011	572.888	362
1997	1.270.475	406.683	320
1998	888.388	196.088	221
1999	1.157.661	437.873	378
2000	1.190.986	485.653	408
2001	1.128.469	270.335	240
2002	1.475.894	490.405	332
2003	1.467.947	536.565	366
2004	1.419.702	467.837	330
2005	1.258.236	436.727	347
2006	1.428.626	614.185	430
2007	1.427.626	424.772	298
2008	1.518.519	655.560	432
2009	1.630.606	575.278	353
2010	1.299.789	415.354	320
2011	1.687.304	782.966	464

Trabalhos usando a metodologia de biobalística em feijão-caupi ainda estão em fase inicial de desenvolvimento, envolvendo protocolos de

transformaçãoestabelecidos ao longo dos últimos anos, os quais levam em conta a estabilidade do sistema (IVO *et al.*, 2008). Acredita-se que nos próximos anos aparecerá um grande número de trabalhos visando a produção de indivíduos com características que possam ajudar a melhorar a produtividade da cultura.

3.1.1 BRS Tumucumaque

Nos últimos anos, principalmente com a formação dos blocos econômicos como o Mercosul (Mercado Comum do Sul), pode ser notado um crescimento considerável das exigências de qualidade dos produtos agrícolas. A escolha de um produto pelo consumidor está baseada, principalmente, nos atributos visíveis e na presença e extensão de danos físicos (LIMA *et al.*, 2000).

De acordo com Freire-Filho*et al.*(2011), em determinados países, as características comerciais que contribuem para compor o aspecto visual do grão, como a cor do tegumento, o hilo e os caracteres relacionados ao hilo, podem influenciar no preço do produto. Ainda segundo os autores, no Brasil, há uma preferência pela classe Branco, hilo e anel do hilo pequenos, além de membrana e anel do hilo de cor clara.

Em relação aos aspectos visuais da semente, a cultivar de feijão-caupi BRS Tumucumaque (nome comercial da linhagem MNC99-537F-4)está enquadrada nos aspectos mais solicitados pelo consumidor brasileiro, ou seja, cor do tegumento branco, sem halo, hilo pequeno e cor do seu anel marrom claro (Figura 2). No que se refere às qualidades nutricional e culinária, apresenta um bom teor de proteína, é rico em Ferro e Zinco, tem cozimento rápido e um excelente aspecto visual após o cozimento (VILARINHO *et al.*, 2008).

A planta possuiciclo relativamente curto (65-70 dias) e porte semiereto com ramos consistentes, o que lhe confere um bom nível de resistência ao acamamento, facilitando tanto a colheita manual quanto a mecanizada, sendo, portanto, indicada para o cultivo por agricultores familiares e empresariais. Além disso, a cultivar produz bem em uma ampla faixa de ambientes, em diferentes ecossistemas, evidenciando sua ampla adaptação(VILARINHO *et al.*, 2008).

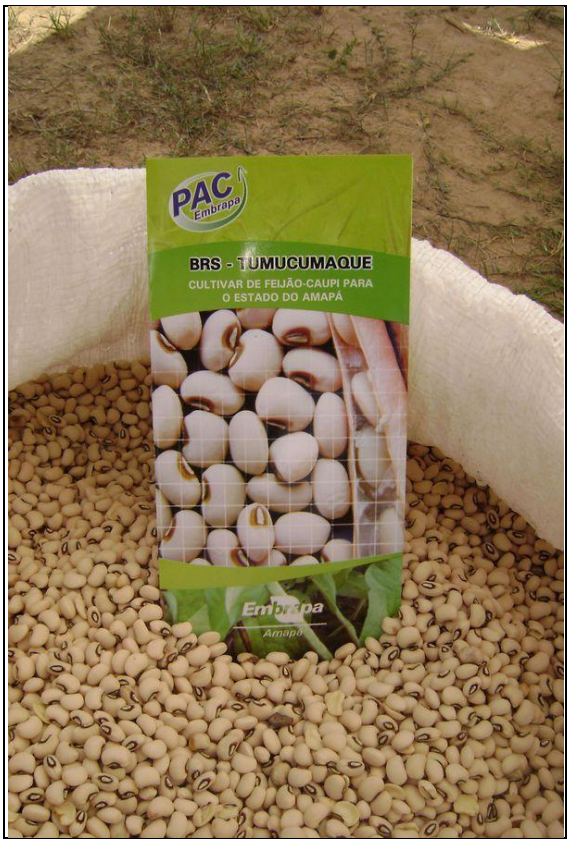

Figura 2 **–** Imagem ilustrativa da cv. BRS Tumucumaque lançada pela Embrapa Meio-Norte, Teresina, PI no ano de 1999. Fonte: Jornal A Gazeta (2013)[2].

3.2Transformação genética de plantas

A capacidade de transferir genes para dentro de qualquer célula, tecido ou organela é um processo fundamental para a revolução genética, juntamente com o *splicing*(processamento do RNA mensageiro), o sequenciamento e a síntese de genes(SANFORD, 1988).Corroborando com Sanford (1988), Vain(2006), em um levantamento detalhado sobre a produção de trabalhos voltados à ciência e tecnologia de plantas geneticamente modificadas nos 30 anos seguintes à primeirapublicação (1973 – 2003), observou que a engenharia genética de plantas compreende uma área bastante dinâmica de pesquisa que passou da fase inicial de desenvolvimento, mas que não atingiu seu limite de saturação. Mais especificamente, trabalhos e publicações que envolvem a tecnologia de plantas transgênicas cresceram substancialmente nos anos 1980 após o desenvolvimento da primeira planta geneticamente modificada, usando tanto vetores binários

[2]Disponível em: <http://www.jornalagazeta-ap.com/portal/?p=2&i=15021&t=Feij%C3%A3o-Caupi_produzido_no_Amap%C3%A1_%C3%A9_destaque_em_Congresso_Nacional>. Acesso em: 29 maio 2013.

(ZAMBRYSKI *et al.*, 1983) como a transferência direta do DNA exógeno (PASZKOWSKI *et al.*, 1984).

A transformação genética de plantas utilizando ferramentas biotecnológicas como a transferência indireta de genes via *Agrobacterium tumefaciens* – método que utiliza uma bactéria que ocorre naturalmente – ou diretamente através do bombardeamento de micropartículas – envolvendo o emprego de meios mecânicos – ou ainda uma combinação de ambos, vem sendo aplicada a mais de 120 espécies de pelo menos 35 famílias de plantas, incluindo aquelas de importância econômica, ornamental e medicinal (MOHAMMED e ABALAKA, 2011).

Em 1984, DeBlock *et al.*(1984) utilizaram a capacidade da *Agrobacterium* de infectar naturalmente locais injuriados em plantas dicotiledôneas causando a formação de tumores (galha) com posterior transferência de um segmento de DNA do plasmídeo (*Tumor inducing* – Ti) chamado de T-DNA (*Transfer*-DNA) para dentro do genoma do hospedeiro(ALIMOHAMMADI e BAGHERIEH-NAJJAR, 2009).

Também foi descoberto que a introdução de um gene exógeno para dentro do T-DNA poderia permitir a sua transferência para o núcleo da célula vegetal, levando ao desenvolvimento da transformação de plantas utilizando uma versão oncogênica, porém desarmada, do plasmídeo Ti que poderia transferir o DNA para plantas sem causar a produção de tumores(MOHAMMED e ABALAKA, 2011).

A *Agrobacterium* infecta naturalmente apenas dicotiledôneas. No entanto, a transferência de genes mediada por *A. tumefaciens* em plantas monocotiledôneas não era possível apenas até recentemente, quando metodologias reprodutíveis e eficientes foram estabelecidas para arroz, milho, banana e trigo(HIEI *et al.*, 1994; MAY *et al.*, 1995; CHENG *et al.*, 1997; ARENCIBIA *et al.*, 1998; ENRIQUEZ-OBREGON *et al.*, 1999).

Para a transformação de plantas, a expressão transiente via *A.tumefaciens* é comumente usada devido à sua alta eficácia e reprodutibilidade, embora limite-se a algumas espécies nãorecalcitrantes, além de necessitar da construção de um complicado vetor binário. Há, ainda, a transferência direta de genes que é bastante útil para qualquer vetor plasmidial e pode ser aplicada em todos os tecidos vegetais, tanto de monocotiledôneas quanto de dicotiledôneas. Contudo, dispositivos especiais são necessários para a entrega de partículas(CHENG *et al.*, 2009).

Um mecanismo de transformação direta de genes ideal tem que cumprir seu papel em três etapas. Primeiro, permitir a transformação de células, tecidos e

espécies já transformadas – mas mais diretamente, de forma mais simples e rápida. Em segundo lugar, possibilitar a transferência de genes para as numerosas células, tecidos e espécies para os quais nenhum outro método foi ainda definido. Em terceiro lugar, permitir a utilização de um único aparelho (e o mesmo protocolo de base) para todos os experimentos de transformação genética(SANFORD, 1988).

Visando minimizar os problemas que acometem a cultura do feijão-caupi, já foram estabelecidos diversos protocolos na área de engenharia genética com objetivos diferentes, mas todos tendo como base o empenho na produção de exemplares tolerantes aos estresses inerentes às áreas de produção (Quadro 1).

Quadro 1 – Demonstração de alguns trabalhos envolvendo a transformação genética de leguminosas (família Fabaceae) a partir do uso das duas metodologias mais importantes.

Espécie[1]	Método de transformação[1]	Gene	Alvo	Fonte
Phaseolus vulgaris	Biobalística	*gus, neo*	Eixos embrionários	ARAGAO *et al.*, 1996
Vigna unguiculata	*A. tumefaciens*	*Hpt*	Cotilédones	MUTHUKUMAR *et al.*, 1996
V. unguiculata	*A. tumefaciens*	*αAI-1*	Cotilédones	UMAHARAN *et al.*, 1997
Glicine max	Biobalística e *A. tumefaciens*	*Gus*	Eixos embrionários	DROSTE *et al.*, 2000
G. max	Biobalística	*Ahas*	Eixos embrionários	ARAGÃO *et al.*, 2000
G max	Biobalística e *A. tumefaciens*	*gus, hpt*	Cotilédones	DROSTE *et al.*, 2002
V.unguiculata	Biobalística	*Gus*	Eixos embrionários	IKEA *et al.*, 2003
V.unguiculata	Biobalística	*Gus*	Eixos embrionários	IVO *et al.*, 2008
G.max, P.vulgaris	Biobalística	*Ahas*	Eixos embrionários	RECH *et al.*, 2008
G.max	Biobalística	*cry1Ac*	Cotilédones	HOMRICH *et al.*, 2008
V. unguiculata	*A. tumefaciens*	*Gus*	Cotilédones	REVEENDAR e IGNACIMUTHU, 2010

3.2.1 Biobalística

O sistema de bombardeamento de micropartículas foi criado, em parte, para compensar as limitações do sistema de *Agrobacterium tumefaciens*, tornando-se o método mais eficiente de transferência direta de genes (ZIOLKOWSKI, 2007). Nesta técnica, partículas de tungstênio são envolvidas pelo fragmento de DNA que será utilizado para transformar a planta. Estas partículas são propulsionadas a uma alta velocidade ao tecido alvo e o DNA será lançado para dentro da célula, podendo integrar-se ao genoma desta. Para geração de uma planta transgênica através desta técnica é preciso que haja otimização do protocolo, de acordo com o genótipo usado, procedimento de cultura de tecidos e condições de transformação como a distância e a pressão de disparo(RECH *et al.*, 2008).

Portanto, para aperfeiçoar esse sistema de transformação,em cada trabalhoé necessário que se foque nos seguintes aspectos do processo: preparação e tipo da partícula; velocidade do disparo e escolha do material alvo. É preciso, também, chegar a um balanço entre o número e o tamanho das partículas e entre o dano causado e a quantidade de DNA lançado. Um fragmento pequeno de DNA pode levar a uma baixa frequência de transformação.Por outro lado, uma quantidade muito grande de DNA pode levar a um alto número de cópias e rearranjos na construção do transgene(SLATER *et al.*, 2008).

Para o bombardeamento de micropartículas devem ser considerados vários fatores e parâmetros para a transferência bem sucedida do gene (BARAMPURAM e ZHANG, 2011). O primeiro parâmetro biológico inclui a concepção de um apropriado vetor plasmidial circular ou linear ou de um cassete linear de expressão (KIKKERT *et al.*, 2004).

O estado fisiológico do material também influencia a receptividade ao DNA exógeno e a susceptibilidade à lesão que pode afetar negativamente o resultado do processo de transformação. Para a regeneração das plantas transgênicas é muito importante visar células que sejam competentes tanto para a transformação quanto para a regeneração. Além disso, a capacidade das células bombardeadas em regenerar plantas depende do tipo e da concentração do agente de seleção(KIKKERT *et al.*, 2004).

A fase de aceleração de partículas envolve a aceleração das micropartículas já revestidas com a DNA a ser transferido. A precipitação do DNA nas partículas de metal envolve o uso de sais inorgânicos, além dos reagentes orgânicos(ZIOLKOWSKI, 2007).

Na fase de separação, os aglomerados de partículas são acelerados até se aproximarem do tecido a ser transformado, decompondo-se em partículas menores. A terceira e última fase é o processo de desaceleração. Esta etapa tem lugar depois que as partículas entram no tecido a ser transformado. Uma vez que atingem o tecido, elas se movem com uma velocidade decrescente até chegar às células-alvo (ZHANG *et al.*, 2007).

Um diagrama do sistema de aceleração de micropartículas a partir da alta pressão gerada por gás hélioconstruído porRech*et al.* (2008) mostra esquematicamente cada parte do processo de bombardeamento (Figura 3). Em (a), há uma placa de Petri contendo o material vegetalarranjado de forma que o

meristema-alvofique no raio de ação das partículas; (b) aponta a tela de retenção que possibilitará que apenas o DNA com a partícula chegue ao material vegetal; Em (c), a membrana de carreamento contendo o gene exógeno é posicionada e tem a finalidade de lançá-lo ao alvo; (d) mostra a membrana de ruptura que funciona como um acelerador do gás; Já em (e), há o ajuste da pressão ideal de acordo com o materiale a câmara de alta pressão é pressurizada com o gás hélio; (f) indica a válvula solenoide que é ativada para proporcionar a onda de choque do gás hélio;(g) indica o local onde ocorre a propulsão da agulha que irá romper a membrana de ruptura.

Kononowicz *et al.*(1995) foram os primeiros a relatar a tentativa de transformação de embriões de feijão-caupi por transferência direta de genes, mas com resultados insatisfatórios. Subsequentemente, apenas dois estudos foram descritos para a espécie com a aplicação da mesma técnica. Ikea *et al.*(2003) não conseguiram obter resultados que comprovassem a segregação mendeliana dos transgenes, além de não terem reportado uma frequência de transformação e os métodos de regeneração. Contudo, Ivo *et al.*(2008) obtiveram a taxa de 0,9%, dividindo-se as plantas putativamente positivas pelo número total de eixos embrionários bombardeados.

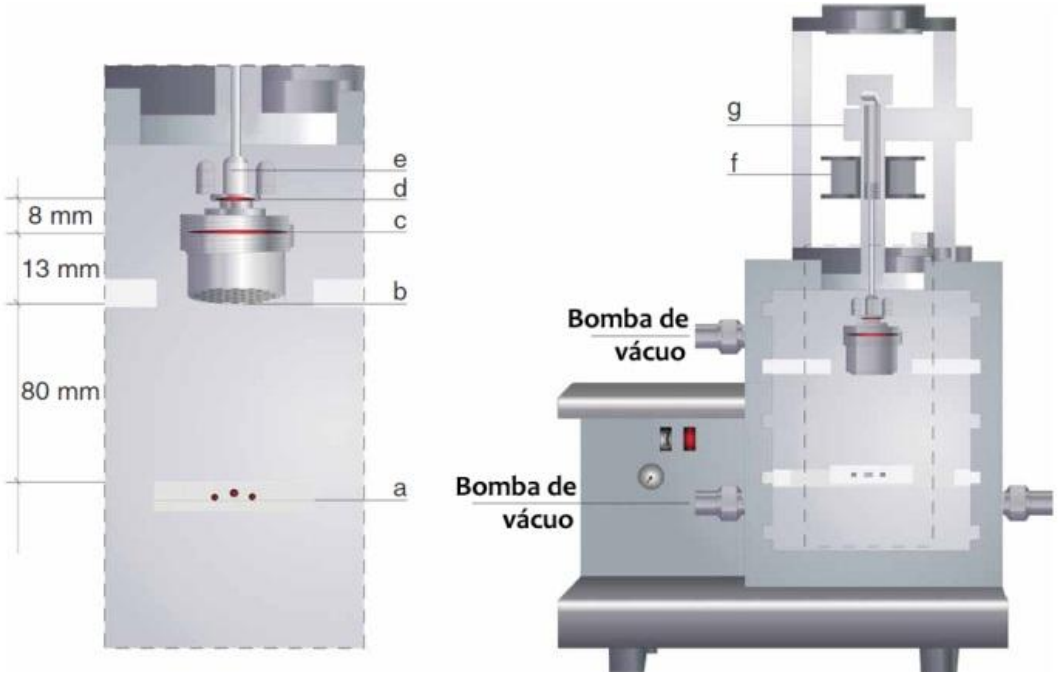

Figura 3 – Esquema ilustrativo do sistema de bombardeamento. Basicamente, a pressão provocada pelo gás hélio chega diretamente à membrana de carreamento e a lança para a tela de retenção que irá segurá-la, permitindo que apenas a micropartícula contendo o DNA atinja a região meristemática dos eixos embrionários. O líquido citoplasmático dissocia o DNA da micropartícula, o qual se integra ao genoma do hospedeiro. Fonte: RECH *et al.* (2008).

Esta tecnologia é limitada devido a alguns inconvenientes, tais como a integração de várias cópias do transgene desejado, além de sequências supérfluas de DNA associadas ao vetor plasmidial eintegrações de multicópias e de sequências indesejáveis que podem conduzir ao silenciamento do gene de interesse na planta transformada (BARAMPURAM e ZHANG, 2011). No entanto, o bombardeamento de micropartículas tornou-se uma ferramenta útil, uma vez que permitiu a direção de genes específicos a um sítio pré-determinado no genoma de uma planta(ZIOLKOWSKI, 2007). Além disso, a biobalística é o único método bem sucedido de transformação disponível para certos genótipos (TAYLOR e FAUQUET, 2002).

3.3 Mutagênese *in vitro*

Todos os organismos sofrem certo número de mutações como resultado de operações celulares normais ou interações com o meio-ambiente. Uma mutação pontual pode ser causada por dois tipos de eventos: modificações químicas do DNA

que mudam diretamente as bases, ou mau funcionamento durante a replicação do DNA causando inserção incorreta de bases. A frequência de mutações varia entre organismos, entre diferentes locos gênicos de um organismo em particular e entre diferentes regiões de um mesmo gene. Cálculos da taxa de mutação são usualmente baseados nas perdas e ganhos de função de um determinado gene (KOVALCHUK *et al.*, 2000).

Em experimentos de mutagênese induzida, independente do agente indutor, é necessário avançar o material tratado por algumas gerações de sementes ou propagações vegetativas. No caso de plantas propagadas por sementes, mutantes recessivos são normalmente selecionados na segunda ou terceira geração após o tratamento. Plantas propagadas vegetativamente necessitam de vários ciclos de propagação para que se obtenham organismos mutados uniformemente ou para reduzir quimeras propiciando, então, a obtenção de mutantes sólidos (AHLOOWALIA & MALUSZYSNKI, 2001).

O fator chave na irradiação de materiais vegetais é a dose a ser utilizada no experimento, a qual é a quantia de energia absorvida pela planta. A unidade de medida da radiação é o Gray (Gy). Um Gray equivale a um Joule de energia por quilo do produto irradiado. Doses de radiação são divididas em três categorias principais: alta (> 10 kGy), média (de 1 a 10 kGy) e baixa (< 1 kGy) (AHLOOWALIA & MALUSZYNSKI, 2001). Em plantas irradiadas a baixas doses de raios gama, há o aparecimento de um grande número de quimeras, observando-se que em tecidos quiméricos células mutadas estão presentes em áreas circunvizinhas às células normais. Durante subsequentes divisões celulares, as células mutadas competem com as normais pela sobrevivência (seleção diplôntica). Caso as células mutadas sobrevivam à seleção diplôntica, as mesmas serão expressas pelas plantas (DATTA *et al.*, 2005).

Outro fenômeno frequentemente encontrado em estudos de cultura de tecidos *in vitro* é o aparecimento de variações espontâneas entre subclones de uma linhagem celular parental (LARKIN & SCOWCROFT, 1981). Os fatores responsáveis por essa variabilidade produzida *in vitro* ainda não estão bem esclarecidos, porém, sabe-se que tal variação é a soma de variações genéticas (mutações cromossômicas e gênicas) que são incorporadas nas plantas regeneradas. Embora existam condições que favoreçam o aumento da variação somaclonal, o que interessa aos melhoristas não é um simples aumento na variabilidade genética, mas

se essa maior variabilidade pode auxiliar na obtenção de novas cultivares mais produtivas ou melhor adaptadas (TULMANN NETO *et al.*, 1998).

Eventos mutagênicos são de particular importância para as plantas, já que qualquer mutação pode ser potencialmente transmitida para as gerações seguintes. Ao contrário dos animais, os vegetais não podem evitar a influência ambiental devido à sua característica séssil (fixada ao solo), necessitando de sistemas dedicados à manutenção da estabilidade genômica (KOVALCHUK *et al.*, 2000).

Em leguminosas, a maioria dos esforços dos trabalhos de mutagênese *in vitro* relaciona-se à fixação biológica de nitrogênio (SAGAN *et al.*, 1994), enquanto a obtenção de mutantes com resistência ou tolerância aos diversos tipos de estresses tem sido relatada com menor frequência (DITA *et al.*, 2006).

Os estudos nessa área têm sido prejudicados devido à recalcitrância à regeneração e à baixa eficiência em encontrar os fenótipos desejados. Todavia, o aprimoramento dos protocolos de regeneração para muitas leguminosas e o desempenho de cultivares produzidas pela indução de mutação indicam que a mutagênese *in vitro* tem desempenhado importante papel no melhoramento destas plantas. A maior dificuldade em técnicas aplicadas à obtenção de mutantes é a grande quantidade de indivíduos necessários para se chegar ao objetivo desejado. Apesar disso, o uso de metodologias de seleção *in vitro* pode minimizar essas desvantagens (DITA *et al.*, 2006).

3.4 Vetores e transformação de plantas

Dois tipos diferentes de vetores – os baseados em vírus recombinantes e os em plasmídeos bacterianos – são usados em transformação genética. Embora vetores baseados em vírus sejam amplamente utilizados e bastante eficientes, os riscos potenciais do gene viral têm que ser seriamente considerados,já os plasmídeos com base em *Escherichia coli*são veículos nãoinfecciosos e versáteis (SOUBRIER *et al.*, 1999).De acordo com Brown (2010), plasmídeos são moléculas circulares de DNA que possuem existência independente na célula bacteriana. Essa molécula quase sempre carrega um ou mais genes e, muitas vezes, esses genes são responsáveis por uma característica útil exibida pela bactéria hospedeira.

Para ser capaz de atuar como um vetor, uma molécula de DNA tem que exibir várias características específicas, mais importante, deve ser capaz de se replicar

dentro da célula hospedeira, de modo que numerosas cópias da molécula de DNA recombinante possam ser produzidas e passadas para as células filhas(BROWN, 2010). Slater *et al.*(2008) listam as propriedades desejáveis para qualquer vetor de clonagem:

1. Ser de tamanho pequeno, não ultrapassando 10 kb, uma vez que plasmídeos menores são mais fáceis de manipular e menos susceptíveis a danos e/ou quebras, nesse caso,também se apresentam, normalmente, em grande número de cópias tendo, portanto, rendimento elevado. Ainda há a vantagem de que a chance de haver outros sítios de restrição não desejados ser menor, fazendo com que seu desenho e sua integração sejam mais simples.

2. Conferir um fenótipo de seleção às células hospedeiras com a finalidade de selecionar apenas as transformadas. A maioria dos vetores plasmidiais carregam genes que conferem resistência a algum tipo de antibiótico, sendo mais comum a ampicilina.

3. Conter sítios únicos para um grande número de enzimas de restrição, permitindo a produção eficiente de vetores recombinantes.

4. Permitir a identificação de colônias de bactérias contendo o plasmídeo recombinante. Esse requisito é normalmente alcançado através do agrupamento dos sítios únicos de restrição em uma pequena área chamada Sítio de Clonagem Múltipla (do inglês *Multiple Cloning Site* – MCS).

Os vetores de transformação de plantas, idealmente, devem ainda fornecer uma rota fácil para a clonagem do gene alvo sob o controle de uma ampla variedade de sequências de promotores e terminadores que tenham a capacidade de expressar simultaneamente vários genes a partir de um único plasmídeo, além de apresentarem uma grande variedade de marcadores de seleção e de genes-repórter (CHUNG *et al.*, 2005).Assim, tipicamente, incluem em seu esqueleto um elemento regulador para a multiplicação bacteriana e, como agente seletivo, um gene de resistência a antibióticos (VIANNA *et al.*, 2011). Ainda, tradicionalmente, o desenho de muitos vetores usados para transformação genética de plantas tem incluído o uso

do 35S CaMV (*Cauliflower Mosaic Virus*) e do nopalina-sintase de *Agrobacterium tumefaciens* (*nos*) – promotor constitutivo que conduz a expressão do gene alvo e marcador de seleção, respectivamente (CHUNG *et al.*, 2005).

Contudo, devido a possíveis problemas como rearranjos nas sequências do transgene ou indesejáveis efeitos negativos sobre a expressão de algum gene endógeno ou, até mesmo, do exógeno, o procedimento mais apropriado para a obtenção de plantas geneticamente modificadas é a utilização de fragmentos lineares do DNA contendo apenas os genes de interesse (VIANNA *et al.*, 2011). No entanto, não há informações suficientes sobre as condições ideais para o uso de cassetes lineares de DNA usados na transformação direta, como na biobalística, por exemplo (CHENG *et al.*, 2009).

Os três trabalhos já relatados de biobalística em feijão-caupi fizeram uso do GUS (β-Glucoronidase) como gene repórter. Kononowicz *et al.*(1995) não relataram o uso de genes de seleção para sua pesquisa. Já Ikea *et al.*(2003), de forma inovadora, relataram um sistema viável de transformação através da biobalística, no entanto, com uso de outros genes exógenos além da característica de interesse. Eles usaram um plasmídeo contendo o gene repórter *uidA* que codifica a β-glucuronidase e um gene marcador de seleção (*bar*), que codifica a fosfinotricina-acetiltransferase (glufosinato), uma enzima que confere resistência ao herbicida biolaphos, ambos dirigidos pelo promotor CaMV35S.

Visando minimizar os efeitos danosos na transformação direta, Aragão *et al.*(2000) desenvolveram um vetor circular que não possui genes de resistência contra antibióticos para a produção de soja (*Glycine max*) transgênica. Este sistema contém um gene mutante isolado de *Arabidopsis thaliana* (*ahas*) utilizado para selecionar células meristemáticas transgênicas após sua introdução, tendo como principal característica a resistência ao Imazapyr que se concentra na região apical da planta, ou seja, a mesma área usada para a inserção do gene de interesse.

Ivo *et al.*(2008) obtiveram sucesso na sua frequência de transformação devido ao fato de terem desenvolvido um sistema de bombardeamento de micropartículas revestidas com o DNA exógeno para a introdução do gene nas células do meristema apical em combinação com o herbicida Imazapyr, baseando-se nas descobertas de Aragão *et al.* (2000).

De acordo com Aragão *et al.*(2000),não há nenhum fator limitante na metodologia supracitada que possa limitar sua ampla utilização em qualquer sistema

de transformação utilizando a seleção de células do meristema apical. A disponibilidade de vetores convenientes pode ser útil para uma variedade de aplicações na biologia molecular vegetal (LEE *et al.*, 2007).

3.5 Interação planta-patógeno e genes de defesa

As plantas são constantemente expostas a uma grande variedade de organismos potencialmente patogênicos como vírus, fungos, bactérias, protozoários, micoplasma e nematoides, além de serem afetadas por condições ambientais adversas (CASTRO e FONTES, 2005). Dessa forma, desenvolveram-se para viver em ambientes onde são expostas a diferentes fatores de estresse. Sendo sésseis, elaboraram mecanismos específicos que lhes permitem detectar mudanças ambientais precisas e responder a condições complexas de estresse, minimizando os danos ao mesmo tempo em que conservam recursos valiosos para o seu crescimento e reprodução (ATKINSON e URWIN, 2012).

Contudo, as respostas de defesa aos ataques bióticos são extremamente variáveis devido à relação coevolutiva entre plantas e seus agentes danosos. Perante esta diversidade, é notável que o ataque biótico desencadeie uma redução uniforme e regulada na transcrição de genes nucleares (BILGIN *et al.*, 2010). Além disso, as diversas classes de patógenos enviam moléculas eficazes para dentro das células vegetais, melhorando, assim, o desempenho microbiano (JONES e DANGL, 2006).

Essa interação planta-patógeno é um mecanismo bem conhecido que envolve a ativação de uma cascata de sinais que, por vezes, resulta em uma rápida resposta de defesa contra uma grande variedade de agentes. Essa acelerada resposta ajuda a planta hospedeira a evitar futuras infecções (GURURANI *et al.*, 2012). Assim, evidências apoiam a noção de que as vias de sinalização de plantas consistem de redes elaboradas, permitindo que as plantas regulem a resistência a doenças (FUJITA *et al.*, 2006).

Por outro lado, agentes patogênicos bem sucedidos têm desenvolvido proteínas que podem suprimir a resposta imune do hospedeiro. No geral, esse processo é altamente evoluído e cuidadosamente monitorado tanto pela planta quanto pelo microrganismo (DODDS e RATHJEN, 2010).

As tentativas de aumentar a produção de alimentos sofrem diversas restrições, das quais o ataque de patógenos às plantações no mundo inteiro antes ou depois da colheita figura entre os principais fatores limitantes (CARVALHO ADE e GOMES, 2009). Analisando a diferença entre o rendimento máximo e o rendimento médio das culturas, observa-se que a maioria das plantas, devido à produção em ambientes desfavoráveis, não atinge seu completo potencial genético (ATKINSON e URWIN, 2012).Assim, genes associados à defesa contra doenças constituem recursos valiosos que devem ser utilizados de uma forma que maximize, em longo prazo, o ganho para a produtividade da cultura (LO IACONO *et al.*, 2012).

Vários mecanismos de defesa contra agentes microbianos têm sido desenvolvidos pelos diversos tipos de organismos existentes (BROEKAERT *et al.*, 1995); porém, ao contrário do que acontece com os mamíferos, as plantas não têm células de defesa móveis ou um sistema imune somático adaptativo.Em vez disso, contam com um sistema de imunidade inata, o qual possui proteínas relacionadas a patógenos (PR, *Pathogene Related*) – codificadas pela planta, mas induzidas especificamente em situações de ataque de patógenos – que são ativadas como mecanismo de defesa capaz de limitar a ação das infecções (VAN LOON e VAN STRIEN, 1999).Envolve, portanto, uma estratégia de defesa a partir da produção de peptídeos antimicrobianos (AMP, *Antimicrobial Peptides*) e com os sinais sistêmicos provenientes dos sítios de infecção (JONES e DANGL, 2006).

As PRs não se acumulam apenas no local infectado, mas, ao reconhecer um patógeno, são induzidas a uma via sistêmica, desenvolvendo uma Resistência Sistêmica Adquirida – SAR, *Sistemic Acquired Resistance*(VAN LOON e VAN STRIEN, 1999). Dessa forma, sua expressão é mediada, principalmente, por proteínas PRR (do inglês, *Pattern Recognition Receptors*; Receptores de Reconhecimento de Padrão), as quais identificam estruturas moleculares conservadas associadas a patógenos, como os componentes da membrana celular e DNA bacterianos ou proteínas virais (BRANDENBURG *et al.*, 2012).

Numerosos genes de plantas relacionados à patogênese já foram caracterizados e estão sendo eficazmente utilizados em programas de melhoramento. Sendo assim, o desenvolvimento de variedades tolerantes a pragas torna-se uma alternativa a outras medidas de proteção contra doenças como, por exemplo, o uso de pesticidas ou outros métodos químicos de controle. Com isso, os benefícios em usar os genes de resistências incluem a redução eficiente do

crescimento do patógeno, um mínimo de dano para a planta hospedeira e total eliminação da aplicação de pesticidas feita pelos agricultores (GURURANI *et al.*, 2012).

3.6 Peptídeos antimicrobianos (AMP, *Antimicrobial Peptides*)

Os AMPs são agrupados em várias famílias e muitos compartilham características gerais, como uma carga global positiva, a presença de pontes dissulfeto (as quais estabilizam a estrutura) e canais iônicos (HAMMAMI *et al.*, 2009). Outra característica peculiar é que, sendo produto de genes de cópia única, os peptídeos antimicrobianos podem ser sintetizados de uma forma rápida e flexível.Devido ao seu pequeno tamanho, podem ser produzidos a partir de um hospedeiro usando uma quantidade mínima de energia e biomassa (BROEKAERT *et al.*, 1995). Portanto, os AMPs fornecem estratégias inovadoras não só para a medicina, mas podem, potencialmente, aumentar o rendimento agrícola através do controle dos fitopatógenos ou pragas (PESTANA-CALSA e CALSA-JR, 2011).

O aumento da resistência bacteriana aos antibióticos convencionais ameaça o futuro de muitos antibióticos atualmente usados e torna a aplicação recorrente de pesticidas na agricultura, representando risco ao meio ambiente e à saúde humana (BELARMINO *et al.*, 2010). A coexpressão das proteínas antimicrobianas com diferentes alvos bioquímicos, por sua vez, é uma abordagem atraente para o desenvolvimento de resistência durável a doenças e contra fitopatógenos (JHA e CHATTOO, 2010).

Embora o reconhecimento e a caracterização de peptídeos antimicrobianos em plantas ainda estejam na sua infância, a interação planta-microrganismo é o mais estudado dos processos devido à sua importância, visando não só entender as associações simbióticas dentro do grupo, mas também dos mecanismos de defesa inata em plantas superiores (BELARMINO e BENKO-ISEPPON, 2010), bem como sua aplicação na biotecnologia e agricultura, devido à demanda e impacto social (PESTANA-CALSA e CALSA-JR, 2011).

3.7 Defensinas vegetais e expressão heteróloga

É conhecido que as defensinas, assim como outros tipos de peptídeos antimicrobianos, são importantes produtos de proteção das plantas(PADOVAN *et al.*, 2010a), sendo expressas em tecidos que promovem a primeira camada de defesa contra pestes e patógenos, tendo efeito antimicrobiano direto tanto contra fungos quanto contra bactérias (LAY e ANDERSON, 2005).

Apresentando entre 45-54 aminoácidos, surpreendentemente, há grandes variações nas suas sequências, embora suas estruturas sejam fortemente conservadas entre plantas, invertebrados e vertebrados (THOMMA *et al.*, 2002). Modificações na sua conformação contribuem para uma grande diversidade de atividades biológicas, variando de inibição da enzima até o bloqueio dos canais de íon (LAY e ANDERSON, 2005), tornando-as bastante versáteis em sua expressão e, consequentemente, na sua aplicabilidade.

Exemplo disso é o fato de que estudos recentes têm mostrado que defensinas de plantas podem penetrar células fúngicas e interagir com alvos intracelulares (MARQUES *et al.*, 2009); outras interagem na membrana plasmática com componentes específicos de fungos, o que resulta na permeabilização desta, tornando-se, assim, uma potencial e atrativa fonte terapêutica no tratamento de infecções causadas por esse tipo de microrganismo. Acredita-se, também, que novas defensinas mais potentes podem atingir certos microrganismos mais especificamente que os antibióticos, incluindo linhagens atualmente resistentes (BELARMINO *et al.*, 2010).

As defensinas de plantas possuem atividades biológicas atrativas à engenharia genética. Esses atributos representam uma vantagem para as abordagens transgênicas pelo fato de um único peptídeo ter o potencial de barrar diversos patógenos (CARVALHO ADE e GOMES, 2009), portanto, podem ter sua característica de resistência utilizada para a produção de culturas transgênicas, resultando em indivíduos mais tolerantes a ataques de organismos patogênicos(PORTIELES *et al.*, 2006).

Na agricultura, o controle por meio de produtos químicos, como os agrotóxicos, aumenta significativamente o custo de produção, bem como a carga maléfica ambiental. Assim, organismos geneticamente modificados que aumentem a tolerância contra as diversas pragas trariam vantagens significativas na redução da

perda de colheitas (JHA e CHATTOO, 2010).A superexpressão dos genes que codificam defensinas, por exemplo, oferece uma abordagem adequada para o controle de doenças que afetam a produtividade da cultura (SWATHI ANURADHA *et al.*, 2008).

Desde a primeira defensina de planta isolada (MENDEZ *et al.*, 1990), o número de genes desse tipo identificados tem aumentado substancialmente, especialmente os encontrados nas espécies da família Fabaceae como, por exemplo, *Phaseolus vulgaris*, *Vicia faba*, *Clitoria ternatea* e *Pisum sativum*(DIMARCQ *et al.*, 1990; ZHANG e LEWIS, 1997; ALMEIDA *et al.*, 2000; ANTCHEVA *et al.*, 2006; GAMES *et al.*, 2008).

Mais recentemente, Padovan *et al.*(2010b) usando *primers* de *Phaseolus vulgaris* em *V. unguiculata*amplificaram uma região no genoma do feijão-caupi (chamada PDEF_VIGUN) contendo uma inserção de 150pb em comparação à sequência de referência e sugeriram uma organização putativa da região genômica amplificada com dois éxons separados por um íntron (os 150pb adicionais).A localização do íntron entre os peptídeos-sinal, apesar da diferença na estrutura primária, nesse caso, é uma característica peculiarcompartilhada com outras defensinas já isoladas tanto de *V. unguiculata*(PELEGRINI *et al.*, 2008)quanto de outras plantas como *Phytophthora inflata*(KARUNANANDAA *et al.*, 1994), *Capsicum annuum*(HOULNE et al., 1998) e *Saccharum officinarum* (PADOVAN et al., 2009).

Em plantas geneticamente modificadas, comprovando o potencial desta tecnologia para ajudar a lidar com patógenos, vários genes de defensinas foram integrados e expressos constitutivamente com sucesso como, por exemplo, podem ser citados o tabaco (*Nicotiana tabacum*) e o amendoim (*Arachis hypogaea*) expressando uma defensina de mostarda (*Brassica juncea*).Em razão disso, apresentaram resistência aos agentes fúngicos *Fusarium moniliforme* e *Phytophthora parasitica* pv. *Nicotianae* e aos patógenos *Pheaoisariopsis personata* e *Cercospora arachidicola*, respectivamente (SWATHI ANURADHA *et al.*, 2008). Um gene antimicrobiano também foi introduzido em rosa (*Rosa hybrida*) através da técnica de transferência indireta de genes via *Agrobacterium tumefaciens*(LI *et al.*, 2003). Utilizando a mesma metodologia em arroz (*Oryza sativa*), Jha e Chattoo (2010) obtiveram exemplares que superexpressam o gene de defensina isolado de rabanete (*Raphanus sativus*).

Assim, a transformação genética que visa a transferência de genes de defesa patogênica oferece uma solução eficaz para o desenvolvimento de variedades com características mais adequadas à agricultura (SWATHI ANURADHA *et al.*, 2008).

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Capítulo I – An improved biolistic transformation protocol for cowpea (*Vigna unguiculata*)

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An improved biolistic transformation protocol for cowpea (*Vigna unguiculata*)

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Abstract

The present work describes a detailed protocol for recovery of transgenic cowpea plants using a combination of resistance to the herbicide imazapyr as a selectable marker, multiple shoot induction from embryonic axes of mature seeds and application of biolistics. It involves detailed description of all stages, including plasmid design, preparation cowpea meristems for bombardment, microparticle-coated DNA bombardment of apical meristems, in vitro culture, steps for selection of transgenic plants and verification of expression levels by qRT-PCR. The known transformation frequency using this protocol ranged from 0.9 to 0.7. The described methodology may be achieved within 8-12 months and is suitable for studies of gene function as well as for the production of transgenic cultivars carrying different traits for breeding purposes.

Keywords: genetic transformation, plasmid design, embryo isolation, bombardment, acclimatization.

1 Introduction

As an important legume used for human food, cowpea (*Vigna unguiculata*) is increasing its productive potential. However, the harvested area, production and productivity of this crop oscillate due to climate changes and attack by pests and pathogens (Freire-Filho et al., 2011). Molecular studies aiming the isolation of genes combined with biotechnological tools such as indirect transfer of genes via *Agrobacterium tumefaciens* or directly via microparticle bombardment can contribute effectively in reducing the problems caused by stress (El-Sayed et al., 2007) and has been applied to more than 120 species from at least 35 plant families (Mohammed and Abalaka, 2011).The microparticle bombardment system was created, in part, to compensate the limitations of the *A. tumefaciens* system, making it the most efficient method for direct gene transfer (Ziolkowski, 2007). This method was initially developed by Sanford (1988) for plants recalcitrant to transformation with *A. tumefaciens* (Barampuram and Zhang, 2011), regarding especially non legumes. These studies were important for the emergence of the first wave of evidence that DNA fully active and functional can be delivered by particle bombardment. In addition, it was also shown that plants could be regenerated from cells or tissues expressing the transferred gene (Ziolkowski, 2007).

Several factors and parameters must be considered for successful gene transfer technology using biolistic (Barampuram and Zhang, 2011). The first biological parameter includes designing a circular or linear plasmid vector or a linear expression cassette (promoter-gene-terminator). It is important to associate the promoter and/or other regulatory sequences with the plant tissue, so that the gene will be expressed at desired levels (Kikkert et al., 2004).

Other biological parameters include the type of tissue, cell size, cell culture age, stage of mitosis, general cell health, the tolerance of the subject to the vacuum, cell density and turgor pressure of the cell. The physiological state of the material influences the responsiveness to exogenous DNA and susceptibility to injury that may adversely affect the outcome of the transformation process. For regeneration of transgenic plants is very important to obtain cells that are competent for transformation so as to regeneration. In addition, the ability of cells to regenerate bombarded plants depends on the type and concentration of the selection agent (Kikkert et al. 2004).

The particle acceleration phase involves acceleration of microparticles coated with DNA that will be transferred. The precipitation of the DNA in the metal particles (tungsten, gold or platinum) includes the use of inorganic salts such as calcium chloride and organic reagents such as glycerin, ethanol and spermidine (Ziolkowski, 2007).

In the separation step, the agglomerates of particles are accelerated till to approach the tissue to be transformed, decomposing into smaller particles. The third and last phase is the process of deceleration. This step takes place after the particles enter the tissue to be transformed. Once the tissue is reached, they move with a decreasing speed until it hits the target cells (Zhang et al., 2007).

This technology is also limited by several drawbacks, such as the integration of several copies of the desired transgene, and redundant sequences associated with the DNA plasmid vector. By the other hand, multicopy integrations and undesirable sequences, may lead to the silencing of the target gene into the transformed plant (Barampuram and Zhang, 2011). However, microparticle bombardment has become a useful tool since it enabled the direction of genes to a

specific predetermined site in the genome of a plant (Ziolkowski, 2007). Furthermore, biolistic is the only successful transformation method available for certain genotypes (Taylor and Fauquet, 2002).

The present article aims to present an improved protocol for cowpea transformation using biolistic applicable to cowpea, contributing to plant transformation works that intent to introduce any desirable characteristic.

2 Experimental design

This protocol was developed for different cowpea cultivars (see Ivo et al., 2008) and was specifically tested with the cultivar BRS Tumucumaque (trade name of the lineage MNC99-537F-4), but may be applied with minor modifications to other cowpea varieties. The microparticle acceleration system presented here made use of a mutant gene isolated from *Arabidopsis thaliana* (*ahas*) used to select transgenic meristematic cells after its introduction, and that the main feature is the resistance to Imazapyr, an herbicide molecule able to translocate systematically, focusing on the plant apical region, in other words, the same area used for insertion of the gene of interest (Aragão et al., 2000).

The preparation phase, before the bombardment, follows the script below:

(1) Construction of a plasmidial vector containing the gene of interest to be inserted and the selectable marker *ahas* gene.

(2) Elaboration of a culture media for the selection of the transformed plants and a bombardment medium for positioning and preservation of the embryos with exposed meristem.

(3) Tungsten microparticle preparation to stock.

(4) BAP (6-Benzilaminopurine) and Imazapyr preparation to stock.

3 Procedures

3.1 Plasmid construction

Specified restriction sites (*NcoI*, *SacI* and *XbaI*) were added to the gene of interest (GI), and this sequence (GI + restriction sites) was inserted in a pBlueScriptSK+ (Stratagene) commercial vector. After addition of the plasmid, the sequence was isolated along with the restriction sites and cloned into a second vector, the pAHAS, which included the *ahas* promoter and a terminator (Figure 1). In this phase, some restriction sites (i.e. *FspI*) can be used to remove the ampicillin resistance gene before particle bombardment, avoiding problems concerning biosafety.

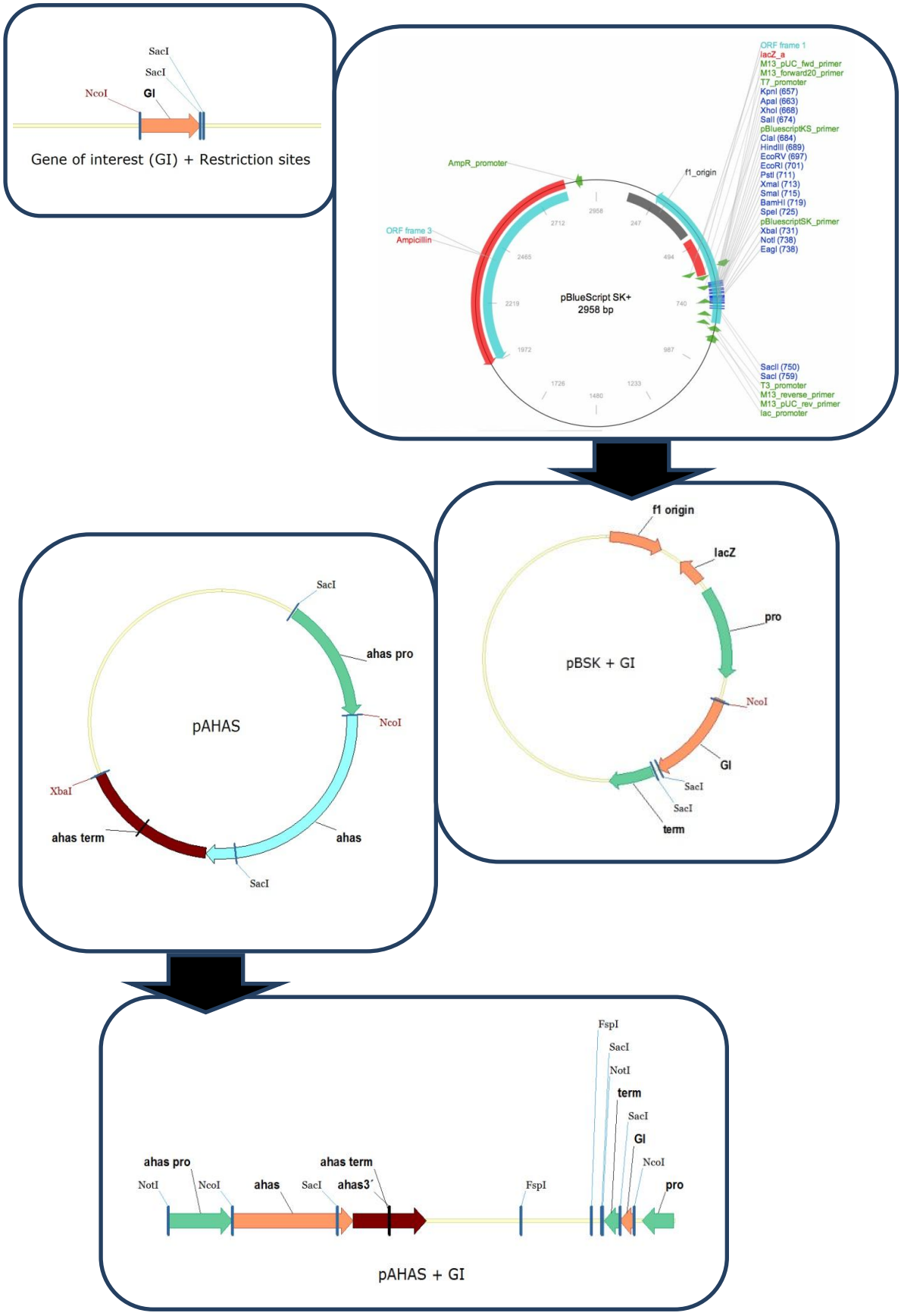

Figure 1. Workflow presenting plasmid construction steps.

3.2 Bombardment

A general workflow of the procedures carried out is presented in Figure 2, with a detailed description as follows.

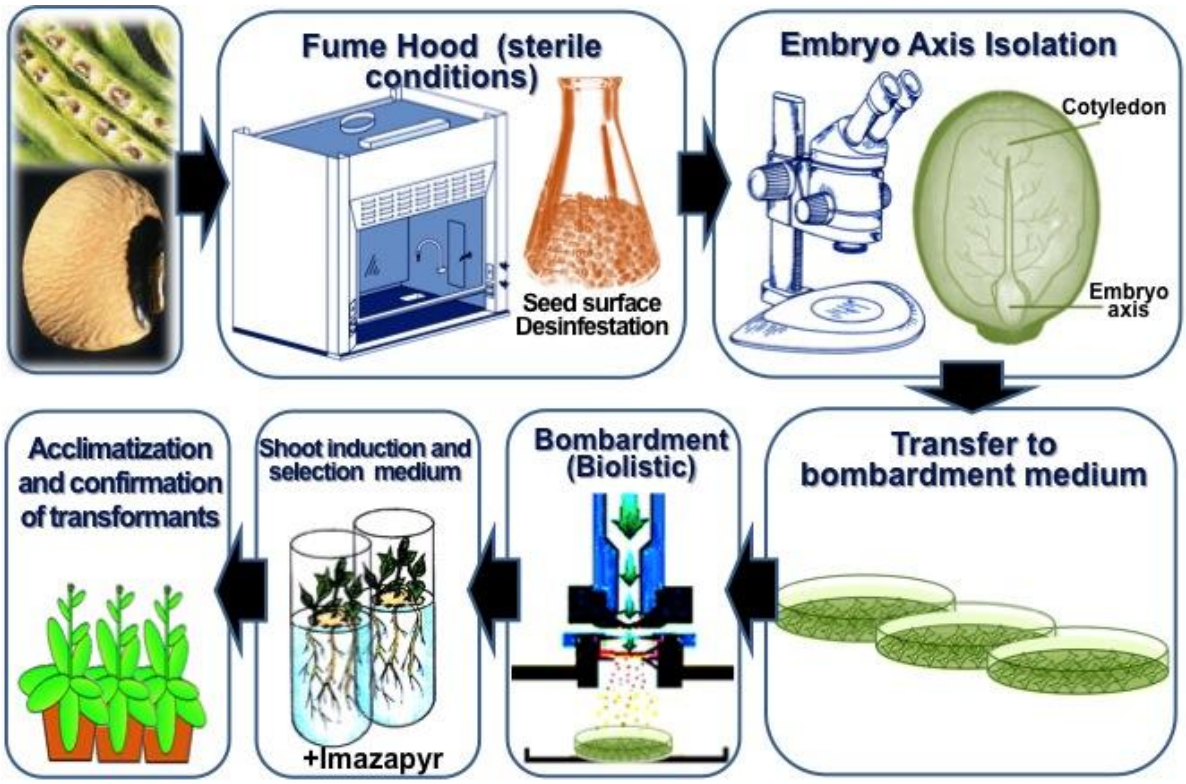

Figure 2. Workflow presenting steps applied, from explants obtaining until transformant recovering.

3.2.1 Preparing the meristem

Initially, the seeds were desinfested with 70% alcohol for one minute, followed by 1% sodium hypochlorite for 20 minutes and washed five times in sterile distilled water. Finally, the seeds remained for 18 hours in the last water to swell.

In order to expose the apical meristem to bombardment, the embryonic axes were isolated from the cotyledons and their primary and primordial leaves were excised in order to expose the apical meristem to bombardment (Figure 3).

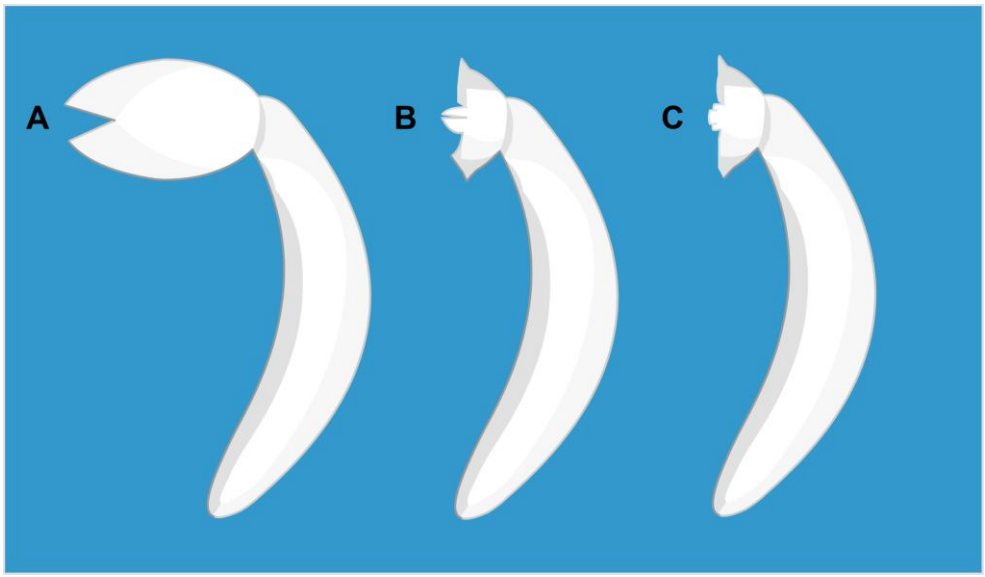

Figure 3. Illustration of the different stages of meristem exposure. **A.** The embryonic axis (still including the primary leaves). **B.** After excision of the primary leaves, exposing the primordial leaves. **C.** After removal of the primary leaves, the apical meristem (central) is completely exposed.

Cowpea is a very difficult crop to work because, besides the primary leaves, the species presents an almost transparent primordial leaves that also need to be removed with extreme caution to avoid apical meristem damaging.

3.2.2 Coating the microparticle by DNA

The previously prepared tungsten particle (supplementary protocol 1) was sonicated for five minutes. In a sterilized blank 1.5 mL centrifuge tube 50 µL of tungsten microparticles were added to 5 µL of plasmid vector in a concentration of approximately 1,000 $ng.mL^{-1}$, 50 µL of $CaCl_2$ and 20 µL of spermidine (supplementary protocol 2). It is very important that the spermidine is the last component to be added, because of its photosensitivity.

The mixture was subjected to a vortex for 10 minutes (at the slowest speed) and centrifugated at 14,500 rpm for 15 seconds. The supernatant was discarded and

replaced by absolute ethanol (150 µL) and centrifugated at 14,500 rpm for 15 seconds. The supernatant was discarded again and the absolute ethanol step was repeated twice.

A total of 24 µL of absolute ethanol was added, mixed gently on the bottom of the tube and 3.2 µL of the precipitated particles were pipetted on the central region of each carrier membrane (six per preparation) already positioned in the bombardment disc. Finally, they were placed in Petri dishes containing silica gel and taken to the desiccator for 20 minutes. It is important that this step is carried out only after exposing meristems.

3.2.3 Bombardment

The exposed embryonic axes were arranged in a Petri dish containing medium suitable for bombardment (supplementary protocol 3) and positioned equidistant on the plate forming a circle and with apical meristem directed upward and towards the center (Figure 4).

The axes bombarded with the DNA coated microparticles were placed in the biolistic equipment, according to the manufacturer's instructions (Figure 5).

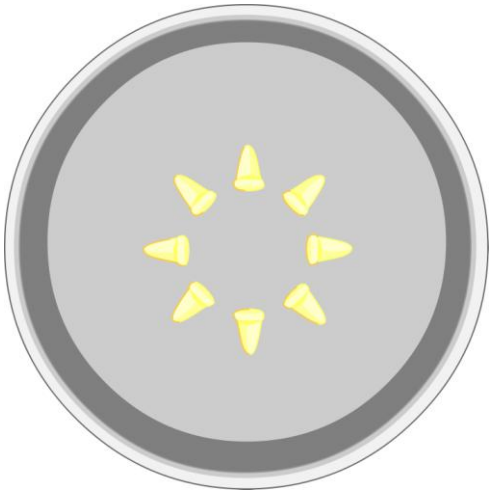

Figure 4. Schematic representation of the recommended embryo disposition for the gene bombardment in cowpea. Adapted from Rech et al., 2008.

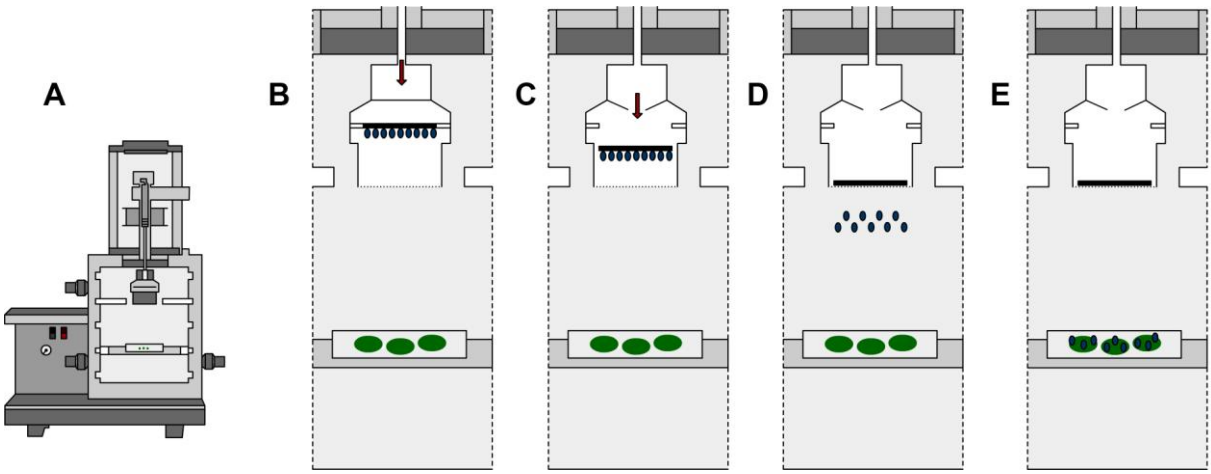

Figure 5. A diagram of the microparticles acceleration system from the helium gas high pressure showing the parts process of each bombardment part. **A.** Lateral cutting of the equipment (adapted from Rech et al., 2008). **B.** A needle breaks the rupture membrane that will accelerate the helium gas speed. **C.** The carrier membrane containing the microparticles coated by the exogenous gene is thrown through the stopping screen. **D.** The stopping screen only allows the microparticles coated by the plasmid to be inserted to pass through it. **E.** The DNA reaching the target tissue.

3.2.4 Culture and selection of transgenic plants

Immediately after bombardment, the axes were transferred to the culture medium for selection and elongation (supplementary protocol 4, 5 and 6) at 28°C with 16 hours photoperiod for approximately 15-20 days.

3.2.5 Acclimatization

The plants that pass the selection process and resist to the medium containing Imazapyr, were first transferred to culture medium containing active charcoal (supplementary protocol 7), as suggested by Ivo et al. (2008), with the objective of removing the selective agent and inducing plant elongation, thus, enabling the development of roots.

After roots emergence, the tolerant specimens were taken for acclimatization as proposed by Ivo et al. (2008) including following steps: (a) soon after the opening of the bottles, possible transformed plants were sprayed with water to prevent dehydration since their stomata are generally open in tissue culture conditions; (b) when withdrawn from the culture medium, roots were washed gently to avoid risks of contamination in the soil; (c) transferred to substrate containing vermiculite which inhibits the action of any toxic compound on soil and; (d) coverage of the plants by a wet transparent plastic bag.

Subsequently, the incubation system was taken in a developing room at 25°C and 16 hours photoperiod, until the plants recover from the transfer shock and develop to a point where they were ready to be grown under green house conditions.

3.3 Molecular analysis

3.3.1 Genomic DNA extraction

Genomic DNA was extracted from fresh leaves, using the Miniprep-CTAB (cetyl trimethylammonium bromide) method as described in Doyle & Doyle (1987). Approximately 150 mg of plant tissue was macerated in 1.5 mL tubes with 600 µL of CTAB extraction buffer. The mixture was subjected to the temperature of 60° for 20 minutes. Thereafter 400 µL of chloroform/isoamyl alcohol (24:1) was added to the samples and centrifugated at the full speed for five minutes (25°C). The supernatant recovered was transferred to a new 1.5 mL tube and 1.5 mL of isopropanol was added in the same amount of the volume collected of the plant material (about 200 µL).

To precipitate the DNA, another centrifugation was performed under the same conditions above for 10 minutes, the liquid phase (supernatant) was discarded and the pellet washed with 70% ethanol. A final centrifugation was carried out for another five minutes, the alcohol was discarded and the DNA was air dried. Finally, the pellet was resuspended in 20 µL of autoclaved Milli-Q water and stored at 20°C.

3.3.2 Amplification of the inserted gene

Amplification reaction was carried out using 1.5 ng of genomic DNA, 1x PCR buffer, 2 mM $MgCl_{2,}$ 0.6 mM dNTP-mix, 2 mM of each primer, 1U of Taq DNA polymerase and the final volume was adjusted to 25 µL.

The amplifications were performed in a thermocycler and the PCR program with an initial denaturation of 5 min at 95°C followed by 35 cycles including 1 min denaturation at 95°C; 1 min annealing at 58°C; extension at 72°C for 2 min and a final extension step of 7 min at 72°C. The amplification products were separated by electrophoresis on horizontal agarose gel (1%), stained with ethidium bromide and visualized on an ultraviolet transiluminador prior to photographic documentation.

3.4 Confirmation techniques

PCR is generally the first technique used to verify the exogenous gene integration in the genome of the host plants. In addition, there are other widely useful techniques to observe the integration as well as the expression of the inserted gene, with emphasis on Real Time Quantitative PCR (RTq-PCR), Acetolactate Synthase Activity (ALS) and Southern Blot.

Real Time Quantitative PCR (RT-qPCR)

Ingham et al. (2001) demonstrated that Real Time Quantitative PCR provide a fast method that can be used to determine transgene copy number in transformed plants and could easily be automated to a large number of samples (Gachon et al., 2004). The details and requirements necessary to obtain reliable data using this technique were reviewed by Bustin (2002).

Recently, in their work about gene expression induced by abiotic stress in nodules of cowpea, Silva et al. (2012) adjusted the RT-qPCR protocol for this species. According to them, the RT-qPCR reactions were performed with three biological replicates for each treatment. In addition, for each biological repetition

three technical repetitions from the same cDNA dilution (1:50) were executed. The present work adopted the same protocol reported as follows: to 10 µL of the cDNA single strand, 4.3 µL of nuclease free water was added, 2 µL PCR buffer 10x (200 mmol.L^{-1} Tris-HCl pH 8.4 and 500 mmol.L^{-1} KCl), 1.2 µL of $MgCl_2$ (50 mmol.L^{-1}), 0.2 µL of each initiator (10 pmol. μL^{-1}), 2 µL of SYBR Green I nucleic acid gel stain diluted in H_2O (1:10.000) and 0.05 µL of the enzyme Platinum Taq DNA polymerase 5 U. μL^{-1}. After the enzyme activation for five minutes (94°C), the reaction proceed for 40 denaturation cycles (94°C) for 15s, annealing at 60°C for 10s e extensions at 72°C for 15s. After each cycle, fluorescence data were collected at 60°C for 35s.

Acetolactate Synthase Activity (ALS)

Acetolactate synthase (ALS) was the other name given to the acetohydroxyacid synthase (AHAS), enzyme inhibited by Imidazolinone herbicides, which is a critical enzyme for the biosynthesis of branched chain amino acids in plants. Also, several mutations in ALS genes confer herbicide-resistance in several plant species (Tan et al., 2005). Sato et al. (2009) reported in their study a protocol applied in transgenic tall fescue (*Festuca arundinacea*), but that can be applied extensively with minor modifications. In the present work leaves (50 mg) were cut into small pieces and incubated in 4 mL of pretreatment solution [25% MS basal medium, 500 µM 1,1-cyclopropanedicarboxylic acid and 10 mM pyruvic acid sodium salt] with or without 0.1 µM BS under fluorescent light at 30°C for 24 h. Only the leaf tissues were transferred to a new tube and frozen for 1 h. Subsequently, 220 µL of 0.025% Triton X-100 solution was added, and the tube heated at 60°C for 10 min. After incubation, 200 µL of the supernatant was mixed with 20 µL of 5% H_2SO_4 and incubated at 60°C for 30min. Then, 100 µL of 5% 1-naphthol dissolved in 2.5 N

NaOH and 100 µL of 0.5% creatine was added to the mixture, and the mixture was incubated at 37°C for 30 min. The color of the reaction mixture was observed, and the absorbance at 530 nm was measured by a spectrophotometer.

Southern Blot

As highlighted by Sambrook and Russell (2001), the Southern Blot has been a classic method to study how genes are organized within genomes by mapping restriction sites in and around segments of genomic DNA for which specific probes are available.

In the present work we applied the protocol used by Ivo et al. (2008) and adapted from Sambrook and Russell (2001), but with specific modifications to *V. unguiculata*. Genomic DNA (15 μ g) was digested with restriction enzymes (e.g. *Nco*I or *Xba*I), separated on 1% agarose gel and transferred to a nylon membrane. In this case, hybridization was carried out using the 5' region of the *ahas* gene (probe a) or the *act*2 promoter (probe b), labeled with $^{\alpha}$32P dCTP (1.13 X 10^{14} Bq mol^{-1}) using a random primer DNA labeling kit according to the manufacturer's instructions. The bands were visualized and photographed with a fluorescent image analyzer.

Concluding remarks

For plant transformation, transient expression via *A. tumefaciens* has been often used because of its high accuracy and reproducibility, although this method has been limited to a few non-recalcitrant species and requires the complicated construction of a binary vector. On the other hand, the direct gene transfer can also be applied in all plant tissues, in both mono and dicots, besides the advantage

associated to the use of any plasmid vector. However, special devices are required to project the particles to the target meristem, as emphasized by Cheng et al. (2009).

The protocol described was applied to two different approaches, one of them aiming to establish the protocol (Ivo et al., 2008) using the *gus* gene under control of the *act2* promoter, and a second one using a defensin gene (Azevedo et al., in prep.). It represents an important contribution to the biotechnological improvement of cowpea and may be applicable in the future for transformation.

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Supplementary content

Supplementary Protocol 1

Tungsten microparticle

Weigh 60 mg of tungsten particles M10 (Atlantic Equipment Engineers) and, in the flow chamber, add 1 ml of alcohol 70%. Stir for 15 minutes by vortexing and centrifuge at 14.500 rpm for five minutes. With the aid of the pipette, carefully discarding the supernatant and add to the precipitate 1 ml of ultrapure autoclaved water. Stir for 10 seconds by vortexing and centrifuge for five minutes at 14.500 rpm. Repeat washing with water and subsequent steps two more times. Resuspend in 1 ml of sterile glycerol 50%. Store the particles at -20°C. To prepare glycerol 50%, add equal parts of autoclaved ultrapure water and glycerol. Finally, autoclave and store approximately at 22°C.

Supplementary Protocol 2

$CaCl_2$ 2.5 M and Spermidine 0.1 M stock solutions

The $CaCl_2$ only must be autoclaved and divided into smaller aliquots for distribution in centrifuge tubes. Store at -20°C.

Dissolve 1 g of spermidine in 68.8 ml of distilled water and filter the solution. Aliquot into 200 uL microcentrifuge tubes and store at -20°C.

Supplementary Protocol 3

Bombardment medium

2.2 $g.L^{-1}$ of MS Basal Salt Mixture without vitamins (Aldrich-Sigma Chemical Co. Ltd.)

8 $g.L^{-1}$ of phytagel

pH 5.8

Supplementary Protocol 4

Culture media

4.4 $g.L^{-1}$ of MS Basal Salt Mixture with vitamins (Aldrich-Sigma Chemical Co. Ltd.)

30 $g.L^{-1}$ of sucrose

6 $g.L^{-1}$ of agar

250 $\mu L.L^{-1}$ of imazapyr

BAP at 5 $mg.mL^{-1}$

pH 5.8

Supplementary Protocol 5

Imazapyr stock solution (1 mM)

Dissolve 13.17 mg of Imazapyr in 50 ml of sterile purified water. Filter and store at -20°C with the tube covered with tinfoil and only add it after autoclaving when the culture medium is at a temperature below 55°C, since this is a molecule photo and thermosensitive.

Supplementary Protocol 6

BAP stock solution (5 $mg.mL^{-1}$)

Dissolve 100 mg of BAP in HCl 1N and complete to 20 ml with distilled water. It is not necessary to filter or autoclaving, since the phytohormone is added to the culture medium before autoclaving. Store at -20°C.

Supplementary Protocol 7

Active charcoal medium

2,2 $g.L^{-1}$ of MS with vitamins (Aldrich-Sigma Chemical Co. Ltd.)

30 $g.L^{-1}$ of sucrose

6 $g.L^{-1}$ of agar

2 $g.L^{-1}$ of active charcoal

Capítulo II – Biolistic transformation of a cowpea plant with a defensin (antimicrobial peptide)

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Biolistic transformation of a cowpea plant with a defensin (antimicrobial peptide)

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Abstract

Cowpea is an important crop especially in dry tropical regions, however, its productivity oscillates due to climatic changes and attack by pathogens and pests. Considering biotic stresses, molecular studies aiming the isolation of genes combined with biotechnological tools, such as transgenesis may achieve a reduction of existing damages and losses. This work aimed to generate a transgenic cowpea plant with increased tolerance to pathogen attack by the insertion of a defensin coding gene (PDEF_VIGUN) from the same species (*V. unguiculata*, cultivar BR14 Mulato) with aid of the biolistic methodology and using previously constructed vectors. About 700 cowpea embryos of the cowpea cultivar BRS Tumucumaque were bombarded, whereas five samples went through the selection stages. Once acclimated, they were analyzed by PCR, allowing the amplification of a 750 bp fragment including the 624 bp coding sequence and flanking regions. The identified transformants are being cloned and will be evaluated regarding morphology, stability and inheritance of the inserted sequence, as well as performance after inoculation by pathogenic agents.

Keywords:*Vigna unguiculata*. Defensin. Transgenic. Biolistic. Genetic engineering.

1 Introduction

Molecular studies aiming at the isolation of genes combined with biotechnological tools, such as transgenesis, can contribute effectively in reducing the problems generated by biotic and abiotic stresses in crop plants (El-Sayed et al. 2007). Such inferences with species of the Fabaceae family such as soybean [*Glycine max* (L.) Merr.] and cowpea [*Vigna unguiculata* (L.) Walp.], may help the yield and also the understanding of the dynamics regarding the introduction of new features of interest in legumes, justifying studies on functional genomics and applications on genetic engineering in the insertion of desirable attributes for different cultivars (Citadin et al. 2011).

Cowpea, as an important legume used for human food and also for feed, being widely cultivated in Africa and South America, but also in other regions like Europe, North America and Asia (Timko and Singh, 2008). However, the harvested area, production and productivity of cowpea oscillate significantly from year to year due to climate variations and biotic stresses (attacks by pests and pathogens) that affect negatively the quality of the production (Freire-Filho et al., 2011).

It is known that all living organisms, whether animal, plant or microorganism, are constantly threatened by pathogen attack. Despite of this threat, the establishment of disease is an exception due to efficient defense mechanisms (Benko-Iseppon et al., 2010). Higher plants possess complex and efficient defense mechanisms against various pathogens that lead to transcriptional activation of a cascade of genes from different metabolic pathways, including those responsible for producing antimicrobial peptides as defensins (Chen et al. 2008; Benko-Iseppon et al. 2010).

A recent review (Citadin et al., 2011) on cowpea GM approaches take into consideration attempts carried out in the last 30 years and highlighted the main constraints concerning especially the regeneration step. Until 2006, dozens of works reported successful transformation but many lacked regeneration or still, after the regeneration, the plants did not acclimatized successfully or survived only for limited time (Aragão et al., 2007; Citadin et al., 2011). The first successful approach was carried out by Popelka et al. (2006) that achieved successful transformation and stable Mendelian segregation of the transgenes to a progeny bearing a transformation frequency of 0.15%, by adding thiol compounds during transfection and co-culture with *A. tumefaciens*. The efficiency of this protocol was further improved by Ivo et al. (2008) for biolistic transformation of meristems using *gus*as a reporter and a novel selection regime based on the use of the herbicide imazapyr. The authors also highlighted significant increase in the transformation efficiency (0.9%), which was not variety specific, as previously proposed.

After the establishment of the efficient protocols mentioned, most works on cowpea genetic transformation regarded the acquisition of insect resistance using different approaches (e.g. Adesoye et al., 2008; Solleti et al. 2008a, 2008b; Higgins et al., 2010). Despite of the progresses in insect resistance pointing to a higher economic importance of GMO cowpea in the future, no attempts have been carried out aiming acquisition of defense against pathogen attack, as it is the case of genes expressing Antimicrobial Peptides (AMPs).

Numerous pathogen defense genes have been characterized and effectively used in breeding programs. Thus, the development of tolerant varieties against pathogens becomes a viable alternative when compared to other approaches for plant protection against diseases, such as defensives or chemical control methods.

Therefore, the benefits of using resistance genes include efficient reduction of pathogen growth, minimum damages to the host plant and total elimination of chemical defensives traditionally used by farmers (Gururani et al. 2012).

In a recent approach, Padovan et al. (2010) isolated, sequenced and characterized a defensin from cowpea, shown to be induced after virus inoculation. Considering the potential of such AMPs for crop biotechnology, the present study intended to transform and overexpress an antimicrobial peptide, AMP from the Defensin superfamily in cowpea using the biolistic methodology proposed by Ivo et al. (2008), aiming to produce a plant with increased pathogen resistance.

2 Materials and methods

2.1 Preparation of exogenous DNA

2.1.1 Vector design

A modified defensin sequence previously isolated by Padovan et al. (2010) called PDEF_VIGUN, containing 150 bp was inserted into a pBlueScript® commercial vector (pBSKHaH11 Stratagene/Addgene), with addition of two restriction sites (*Nco*I and *Sac*I). Thereafter, a vector map was constructed with aid of the software VECTOR NTI® (Invitrogen), replacing the coding sequence of the vector by the defensin sequence (Supplementary Figure 1A) due to similarities regarding restriction sites. The new construction was called VuDEF (Supplementary Figure 1B).

After addition of the plasmid, the sequence was isolated along with the restriction sites mentioned, being cloned into a second vector named pAHAS (Supplementary Figure 1C), which contained the ahas promoter and the terminator,

as described previously by Aragão et al. (2000) for soybean (*Glycine max*) plants, with the advantage of the absence of an antibiotic resistance gene. This system contains a mutant gene isolated from *Arabidopsis thaliana* (ahas) used to select transgenic meristematic cells, being its main feature the resistance to Imazapyr, a molecule capable of translocating and concentrating in the apical region, the same area used for insertion of the gene of interest. The whole set of the coded sequence and the regulatory sequences was called expression cassette pAHASVuDEF (Supplementary Figure 1D). To confirm the construction, an agarose gel was run containing samples of pAHASVuDEF, being subsequently digested with the respective restriction enzymes for each site. After verification of the expected fragment sizes (Table 1), the next step regarding the bombardment of *V. unguiculata* (cv. BRS Tumucumaque) embryos was carried out.

Table 1: Default expected for digestion of the vector pAHASVuDEF.

Enzyme	Number of bands expected	Expected fragment size (bp)
*Nco*I	2	6.864 and 1.595
*Not*I	2	7.248 and 1.211
*Sac*I	3	4.416, 3.736 and 307

2.1.2 Cloning and transformation by electroporation and screening of recombinant colonies

The cloning was performed using aliquots of the vector and *Escherichia coli* electrocompetent cells (Invitrogen). After electroporation, 600 µL of LB culture medium (tryptone 2%, yeast extract and NaCl 5M 0.5%) was added to the cell

suspension, incubated at 37°C during 1 h and subsequently plated on Petri dishes containing solid LB medium supplemented with ampicillin (100 mg/mL). After incubation at 37°C for 16 h, individual transformant cells were transferred to 3 mL of LB medium supplemented with ampicillin (100 mg/mL) and incubated overnight at 37°C under constant agitation (180 rpm). All these steps followed Lacorte and Romano (1998).

2.1.3 Construction of vectors for cowpea transformation

The extraction of the plasmid DNA (Miniprep) was performed using the alkaline lysis method for microplates as indicated by Engebrecht et al. (2001), with minor modifications. Initially, the vector was digested with appropriate restriction enzyme (*Not*I, to isolate only the expression cassette) and incubated overnight at 37°C. The digestions products were submitted to electrophoresis (agarose gel 1%) and the bands representing the cassette were isolated and purified using the Wizard® Genomic DNA Purification Kit, as recommended by the manufacturer (Promega). The fragments were confirmed again on a 1% agarose gel and quantified using a 100 bp DNA Ladder (Invitrogen) as size reference.

In parallel, the digestion, dephosphorylation and ligation of the second vector (pAHAS) were performed as Lacorte and Romano (1998). The digestion was carried out with the same restriction enzyme and under the same conditions, except for the purification that, in this case, was not necessary.

The ligation reaction was performed with 1 µL of the T4 DNA ligase enzyme (Invitrogen), 4 µL of T4 DNA ligase buffer (Invitrogen), 1 µL of the vector pAHAS, 3 µL of the previously isolated cassette and 11 µL of autoclaved Milli-Q water. The

reaction was incubated at 16°C in a thermocycler (MyCycler, BioRad) overnight. The ligation was electroporated, plated on solid LB medium containing ampicillin (100 mg/mL) and grown at 37°C (overnight). Afterwards the plasmid DNA (Miniprep) was extracted from the set “cassette + pAHAS” and a further digestion was carried out, now with the restriction enzymes *Not*I, *Sac*I and *Nco*I to confirm the correct introduction of the insert. The sizes of the expected fragments were confirmed by electrophoresis (1% agarose gel).

2.1.4 Multiplication of the vector by Maxiprep

According to Lacorte and Romano (2008), for multiplication of the generated product, a maxiprep was held and, to start bombardment of the embryos with the correct and checked vector, a digestion of the Maxiprep product was made with the enzymes *Nco*I, *Not*I and *Sac*I (1 µL of enzyme, 1 µL of enzyme buffer, 2 µL of the maxiprep product and 7 µL of autoclaved Milli-Q water). Furthermore, for biosafety issues, it was necessary to inactivate the ampicillin using the enzyme *Fsp*1 (5 E.U./µL), digesting the mixture overnight at 37°C and checking the expected fragments on 1% agarose gel.

2.2 Cultivar used and gene transfer via biolistic

The cultivar chosen was BRS Tumucumaque (trade name of the lineage MNC99-537F-4), a variety that presents white tegument and high commercial value, framed in the most requested aspects by the cowpea national and international consumers (Vilarinho et al., 2008).

For the experiment, exposed apical meristems of embryonic axes were chosen as target. All steps of the bombardment phase as meristem exposure, the microparticle preparation, the particle coating by the exogenous DNA, the bombardment itself, the culture and selection of transgenic plants and acclimatization followed Ivo et al. (2008).

Initially, the culture medium used for selection contained 10 $mg.L^{-1}$ of the phytohormone BAP (6-benzylaminopurine). However, the plants showed an enormous amount of undifferentiated cells (calli). Thus, the concentration of BAP was reduced, but the plants continued to show deficits in development. So it was determined the value of 2.5 $mg.L^{-1}$ as the optimum concentration of BAP for BRS Tumucumaque.

The bombardment was taken in a self designed bombardment equipment developed at Embrapa Recursos Genéticos e Biotecnologia (CENARGEN), also described by Ivo et al. (2008).

2.2.1 Extraction of genomic DNA

Genomic DNA was extracted from BRS Tumucumaque fresh leaves, using the Miniprep-CTAB (Cetyl Trimethyl Ammonium Bromide) method as described in Doyle & Doyle (1987). Approximately 150 mg of plant tissue was macerated in 1.5 mL tubes with 600 µL of CTAB extraction buffer. The mixture was then incubated at 60°C for 20 minutes. Thereafter 400 µL of chloroform/isoamyl alcohol (24:1) were added to the tubes and the samples were brought to centrifugation full speed at 25°C for five minutes. The supernatant was recovered and transferred to a new 1.5 mL tube and 1.5 mL of isopropanol was added in the same amount of the volume collected of the

plant material (about 200 µL) to precipitate the DNA. Another centrifugation was performed under the same conditions above for 10 minutes, the supernatant was discarded and the pellets washed with 70% ethanol for five minutes. A final centrifugation was carried out for another five minutes, the alcohol was discarded and the DNA air dried. Finally, the pellets were resuspended in 20 µL of autoclaved Milli-Q water and stored at 20°C.

2.2.2. DNA amplification

The amplification reactions were carried out using 1.5 ng of genomic DNA, 1x PCR buffer, 2 mM $MgCl_2$, 0.6 mM dNTP-mix, 2 mM of each primer (Table 2) and 1U of Taq polymerase in a final volume adjusted to 25 µL. Amplifications were performed in an Eppendorf Mastercycler Gradient thermocycler and the PCR program used was an initial denaturation step at 95°C for 5 min followed by 35 cycles adjusted as follows: denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 2 min, with a final extension step at 72°C for 7 min. The amplification products were separated by electrophoresis on 1% horizontal agarose gel, stained with ethidium bromide, being visualized on an ultraviolet transiluminador and photographed in Molecular Imager® Gel Doc™ XR System (Bio-Rad).

Table 2: Primers used to confirm the transformation.

Primer	Sequence (5’ – 3’)
AHASP124	ACTAGAGATTCCAGCGTCAC
AHAS500C	GTGGCTATACAGATACCTGG

3. Results and Discussion

All steps of the plasmid construction have been confirmed by the respective restriction enzymes, exhibiting the expected sizes (Table 1). For transformation, 700 embryos were bombarded resulting in an endogenous contamination rate of 23%. From these, 21 plants were observed in full development (3%), and five (0.7%) of the specimens went through all the stages of selection pressure (Figure 1A), reaching acclimation. Thus, the transformation frequency (defined as the total number of putative transgenic plants divided by the total number of embryonic axes bombarded) was 0.7%, a number slightly lower than the 0.9% achieved by Ivo et al. (2008) using the same protocol. All plantlets that developed vigorous roots were acclimatized and transferred to soil (Figure 1B).

Figure 1. **A**. Illustration of plants in full development after gene transfer via biolistic and inoculation in selective medium. **B.** Plants incubated at the development room during the acclimatization step.

The set of primers pAHAS 124 (Forward) and pAHAS 500 (Reverse), allowed the amplification of a 750 bp fragment (Figure 2). Taking into consideration the size

of the primers along with defensin sequence the fragment size obtained was precisely the expected result.

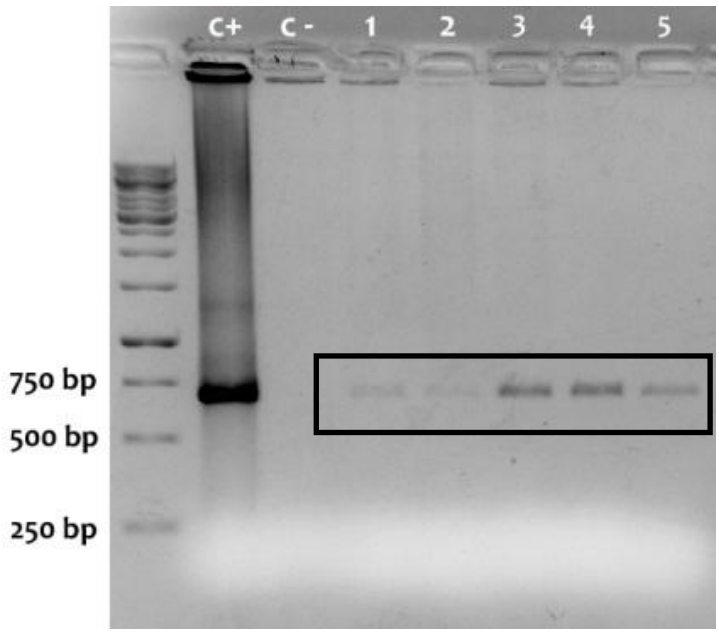

Figure 2. C+. Positive control; C-.Negative control; 1-5. Fragments amplified from the primers AHAS124 (forward) and AHAS500 (reverse), confirming the insertion of the plasmid into the five host plants.

The present work illustrates the huge amount of work necessary for succesfull transformation in cowpea, also observed in some previous reports. Transformation systems via biolistic containing the reporter gene and/or selection marker gene driven by the CaMV 35S promoter in plants of the Leguminosae family like *V. unguiculata*, *Phaseolus vulgaris* and *Glycine max* (Ikea et al., 2003; Aragão et al., 1996; Homrich et al., 2008). A few previous works show efforts in genetic transformation of cowpea. Kononowicz et al. (1995) first reported the attempting to transform cowpea embryos by direct transfer of genes, but with unsatisfactory results. Subsequently, only two studies have been reported for this species with application of the same technique. Ikea et al. (2003) failed to confirm the results of

the GUS positivity by PCR, but Ivo et al. (2008) have obtained the rate of 0.9% of the putatively positive.

Ivo et al. (2008) have succeeded in transformation frequency due to the fact that they have developed a bombardment system with DNA-coated microparticles for the introduction of exogenous gene in meristematic cells in combination with the herbicide Imazapyr, based on the discoveries of Aragão et al. (2000). Therefore, with the same strategy that Ivo et al. (2008), but with addition of the CaMV 35S promoter, we obtained a transformation rate of 0.7%.

Since this is a process that involves the important step of tissue culture, comparisons between rates of specimens development of different works must be done in order to have a quantitative parameter. Azevedo et al. (2007) showed in their assessments of the regenerative potential of different cultivars of *V. unguiculata* varied responses to *in vitro* regeneration both among different cultivars and among tissues of a same cultivar, leading to the belief that phytohormones are genotype-specific, acting variously even within the same species. Fact showed in our results with the variation in the concentration of BAP (6-benzylaminopurine) in relation to other similar studies on cowpea.

The three studies mentioned before have made use of the GUS as reporter gene in addition to the gene of interest. Kononowicz et al. (1995) did not report the use of a gene selection for your research. On the other hand, Ikea et al. (2003) used a plasmid containing the reporter gene *uidA* that encodes the β-glucuronidase and a selection marker gene (*bar*) which encodes the phosphinothricin acetyltransferase, an enzyme that confers resistance to the herbicide Biolaphos. Ivo and coworkers (2008) have established the cowpea transformation protocol via biolistic with stable Mendelian inheritance of transgenes using as selection marker the *ahas* gene.

While previous results have become satisfactory, much has been discussed about possible negative effects of the exogenous genes of GMOs (Genetic Modified Organisms) on human health, animal and the environment. It is understandable that it´s not desirable the presence of other exogenous agents than the feature that wants to express (Miki and McHugh 2004). Thus, stated that in order to obtain transgenic plants, the most appropriate procedure to obtain transgenic plants is the one that makes use of DNA fragments containing only the gene of interest (Vianna et al., 2011). Therefore, we chose to use only the molecule selection Imazapyr, the defensin gene and the promoters and terminators, ensuring the reliability of the product generated regarding biosafety concerns, confirming the results from appropriate molecular techniques.

Several other works using the method of microparticles acceleration coated with DNA and inserted into the apical meristem of the embryonic axis were reported in Leguminosae attempting to optimize the technique, making it easier to run (McCabe et al. 1988; Finer and MacMullen 1991; Christou and McCabe 1992; Aragão 1996; Aragão 2000; Droste 2002).

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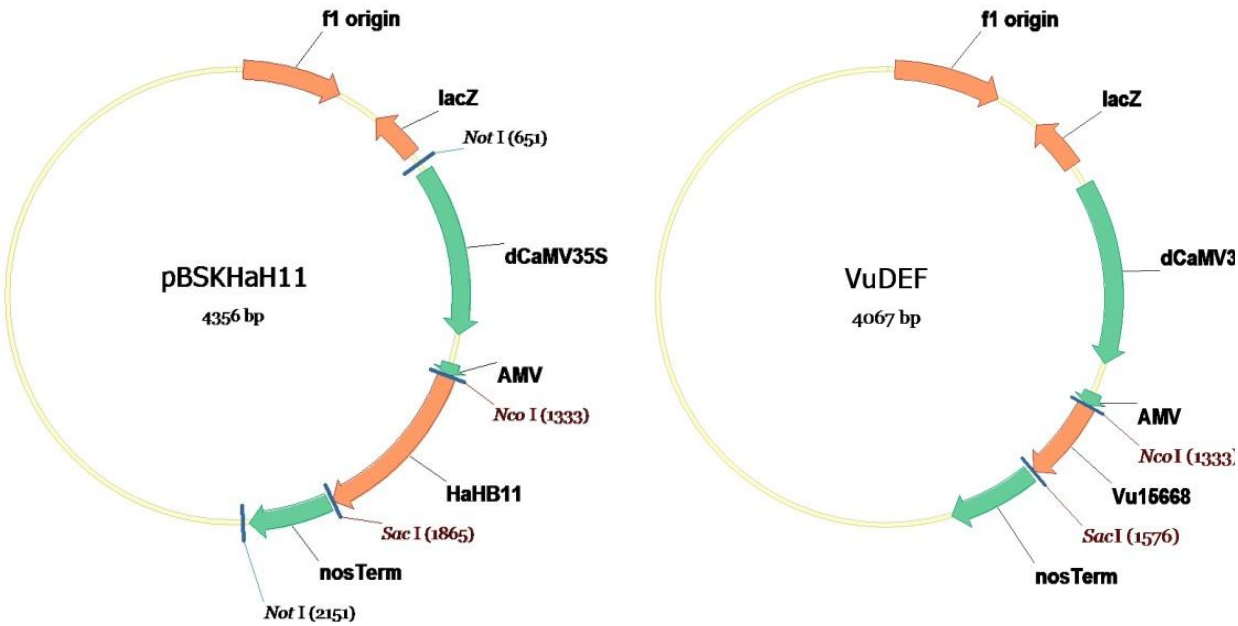

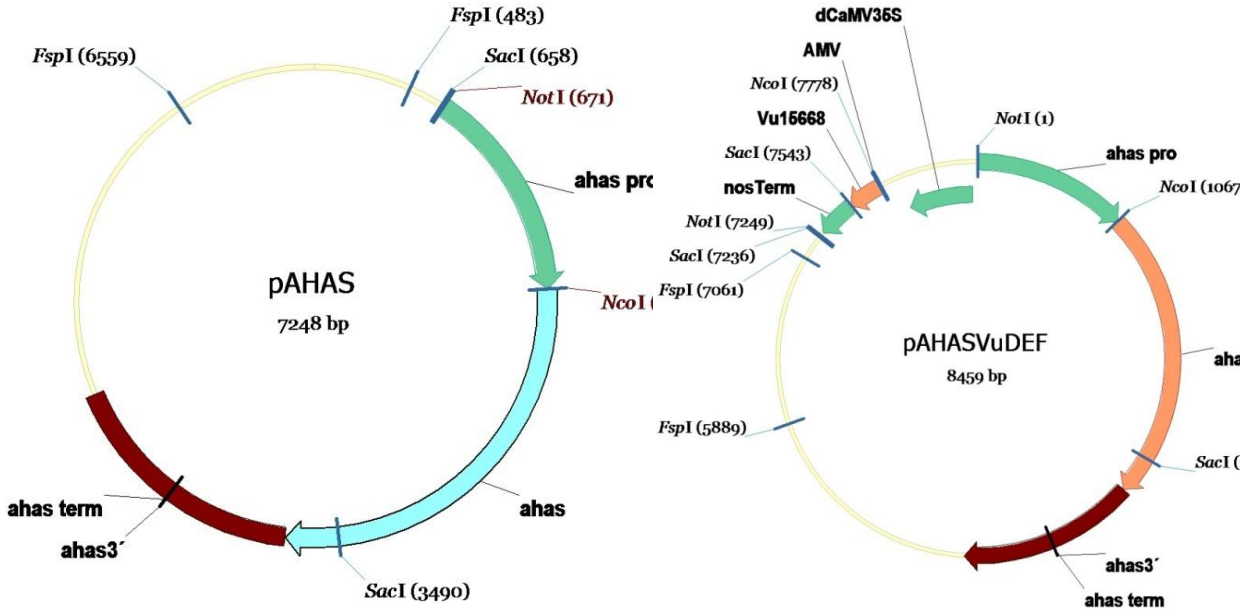

Supplementary Figure S1 – Illustration of vectors adaptations used in the present work. A. The original pBlueScript® vector map, in which the region HaHB11 was replaced by the defensin sequence. B. Vector containing the restriction sites *Nco* I and *Sac* I after insertion of the sequence that encodes defensin (Vu15668). C. Illustrative map of the vector pAHAS containing the restriction sites *Not*I, *Sac*I and *Nco*I. D. Cloning of VuDEF in a second vector, pAHAS that bears the restriction sites *Nco*I, *Not*I and *Sac*I. The 'dCaMV35S' sequence is the promoter and 'nos Term' the terminator of the Vu15668 (VuDEF). The ahas promoter is indicated as 'ahas pro' and the terminator as 'ahas term'.

Capítulo III – Establishment of a protocol for mutation induction by gamma rays and in vitro selection of cowpea (*Vigna unguiculata*) for salinity tolerance
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Establishment of a protocol for mutation induction by gamma rays and in vitro selection of cowpea (*Vigna unguiculata*) for salinity tolerance

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Abstract

Despite of its limited productivity under conditions of soil salinity and drought, cowpea is among the most important protein sources for the rural populations in the dry tropical regions. In the course of this study, attempts were made to evaluate the most promising approaches to induce *in vitro* mutation in cowpea using gamma rays. For this purpose we selected a commercial elite cultivar (BR14-Mulato) that in previous experiments showed increased regenerative potential, but lacks any indication of salinity tolerance. The present approach included a first phase with 10 different trials, involving two tissues (immature embryo axis and cotyledons) and five salt concentrations (0, 172, 258 and 344 mM), aiming to identify the minimum *in vitro* inhibitory NaCl dose. After this approach, the concentration of 172 mM (1%) was considered as the most appropriated, being used for the next trials. In a second phase treatments have been carried out including four different gamma ray exposition intensities (100, 150 and 200 Gy) and negative control (no irradiation) considering two tissues (cotyledons and embryo axis) and with and posterior exposition to salinity selective pressure (medium containing 172 mM NaCl). This last experiment was conducted from randomized design for a total of ten treatments arranged in a 5 x 2 factorial (4 doses of radiation and two levels of NaCl, i.e., in absence or presence of salt), and worked in five repetitions, comprising a total of 40 treatments (each of them comprising 20 cotyledons and 10 embryo axes). Considering the results, the gamma ray intensities of 100 and 150 Gy seem to potentiate the regenerative responses in cultivar BR14-Mulato, whereas the dose of 200 Gy presented more deleterious effects. The next step regards de application of

the developed protocol to a larger number of explants, for obtaining salinity tolerant cowpea plants.

Key words: *in vitro* mutagenesis, gamma Ray, regeneration, abiotic stress.

1 Introduction

Cowpea [*Vigna unguiculata* (L.) Walp.] is among the legumes with highest protein content, being well adapted to the climate and soil of the Brazilian northeastern region, standing out for cultivation under semi-arid regions, where other beans do not grow properly (Leite et al., 1999; Freire-Filho et al., 2011). However, irrigation may be necessary for the plantlet establishment, what may be a concern due to salinity in the irrigation water, the intense evaporation and lack of efficient drainage of the Brazilian northeastern soil, reducing the development of these plants (Dantas et al., 2002).

Modern biotechnology tools have achieved an almost inexhaustible potential for obtaining desirable crop features. An additional unconventional alternative to crop improvement regards *in vitro mutagenesis*, most commonly using ionizing radiation, like X- or gamma rays at very low doses, which usually cause mutations as substitutions, insertions or deletions (Ahloowalia and Maluszynski, 2001). The viability of the technique is well established, as shown by data provided by the FAO/IAEA (Program for Nuclear Techniques in Food and Agriculture, 2013), which recorded over 3,200 varieties obtained by mutation of more than 160 species of plants worldwide.

Explants of several angiosperm species have been successfully cultured *in vitro*, indicating the potential of the technique for obtaining whole plantlets from cells, plant tissues or organs. However, in pulses, *in vitro* regeneration has been given by researchers to frequent limitation due to recalcitrant species, especially in legumes, constituting the primary bottleneck advances in the group (Christou, 1997; Popelka et al., 2004). Despite the difficulties reported, recent examples of success have been reported (revised by Citadin et al., 2011) including stable plant regeneration from

cowpea transformed via *Agrobacterium tumefaciens* (Popelka et al., 2006; Chaudhury et al., 2007) or via biolistic (Ivo et al., 2008).

The present study aimed to develop a protocol for mutant generation of cowpea and *in vitro* selection for salinity tolerance, including the following steps: (I) definition of minimum NaCl concentration that can inhibit the development of seeds and to determine the levels of selection pressure; (II) establishment of a procedure for mutation induction using gamma radiation *in vitro*, with a preliminary assessment of the radiation levels effective for this purpose, and (III) monitoring of the regeneration *in vitro* process of induced and non-induced plants under salt stress.

2 Material and Methods

Plant material and explant isolation

For this study, the cultivar BR14-Mulato was selected due to its satisfactory *in vitro* regenerative potential in previous assessments (Azevedo et al., 2007), and also its interesting features for cultivation in northeastern Brazil. Seeds were provided by Embrapa Meio-Norte (Agricultural Research Center of the Mid-North - CPAMN, Teresina, Piauí, Brazil). Fresh ripe seeds were obtained from plants grown in a greenhouse under 14 hours photoperiod, anti-aphid net and at room temperature.

Seed disinfestation and preparation of solutions were made in sterile laminar flow hood. Initially, seeds were rinsed with distilled water before soaking the seeds in 70% alcohol solution for one minute, followed by immersion for 20 minutes in a solution of 0.2% sodium hypochlorite. The seeds were then washed three times with sterile water, and adding, in the third 500 mg of the antibiotic Cephalexin (2.5 $mg.mL^{-1}$, CEFAGEL®). Seeds remained in this solution in a Petri dish for 24 hours for initial germination.

Subsequently cotyledons and embryonic axes were isolated for inoculation in regeneration medium optimized for cowpea, as follows: salts and vitamins of Murashige and Skoog (1962) supplemented with 30 $g.L^{-1}$ sucrose, 6.0 $g.L^{-1}$ agar and BAP (6-benzylaminopurine) at a concentration of 2 mg L^{-1}. The explants were cultured in a growth chamber with a 14 h photoperiod at 19°C ± 1°C for 30 days under constant monitoring.

Establishment of the Regenerative Potential under Salinity

The NaCl sensitivity test was performed to determine the minimum dose that would provide growth inhibition *in vitro*. Four different media were tested for inoculation of the seeds after isolation of the explants, based on the medium optimized for cowpea, plus sodium chloride (NaCl) at concentrations indicated in Table 1. Two additional culture media were prepared, containing concentrations of 0.6% (103 mM) and 0.3% (51.5 mM), to allow a gradual decrease from the saline condition, without sudden changes during the transfer to culture medium in the complete absence of NaCl.

Table 1. Different treatments used for evaluation of the regenerative potential of cowpea cultivar BR14-Mulato under salinity with respective concentrations of NaCl.

Treatment	NaCl concentration
T_0	0 mM (negative Control)
T_1	172 mM (1%)
T_2	258 mM (1,5%)
T_3	344 mM (2%)

'In vitro' selection of irradiated explants

Irradiated seeds at doses of 25, 50, 75, 100, 150 and 200 Gy were used for explant isolation and inoculation. Half of them were cultivated in cowpea MS medium plus 1% NaCl (selected concentration), in order to select possible materials tolerant to salinity, while the other half was inoculated in the same medium without addition of salt, to be transferred to the selective medium after 20 days.

Experimental design

The experiment was conducted from randomized design for a total of eight treatments arranged in a 4 x 2 factorial (4 doses of radiation and two levels of NaCl, i.e., in absence or presence of salt), and worked in five repetitions, comprising a total of 40 treatments. After collection and decontamination of plant material, the experiment was conducted in two main stages: analysis of regenerative potential under salinity and *in vitro* selection of irradiated materials.

In the first step two cotyledons were inoculated in each flask, where the explants were separated by a distance of at least 2 cm, to avoid competition between tissues. Both tissues (embryonic axes and cotyledons) were inoculated in standard culture media (MS) with different concentrations of NaCl (172, 258 and 344 mM), for a total of four treatments (including the control medium without salt). The material was evaluated on a weekly basis according to their phenotypic characteristics for full four weeks, being adopted as the main characteristics analyzed swelling, necrosis index (including evaluation of color, appearance, possibly tissue necrosis) and callus formation (indirect regeneration) or plant development (direct regeneration).

In the second phase, 2800 seeds were separated into seven groups containing 400 seeds each, including the seeds not irradiated (0 Gy) that were used as control and the seeds subjected to specific dosages of gamma radiation (25, 50,

75, 100, 150 and 200 Gy) by Gammacell 220 Excel pump irradiating® (Co60) available laboratory GAMALAB Department of Nuclear Energy (DEN) at Federal University of Pernambuco. Tissues isolated from seeds irradiated with different doses of gamma radiation were inoculated in culture media with absence and presence of NaCl. The phenotypic analysis of the explants was performed every seven days during four weeks, taking into account mainly rates direct or indirect organogenesis, and indexes of necrosis and swelling.

3 Results

Differences regarding the regenerative potential were observed during the experiments depending on the tissue medium in relation to salinity treatments. A week after inoculation a endogenous contamination (probably of bacterial origin) was observed in 20% of the flasks in the control and in 40%, 40% and 10% of the flasks containing 1%, 1.5% and 2% NaCl, respectively. Among remaining explants of the control (no salt addition) only 10% presented necrosis of the cotyledons, with overall greening (swelling) of the other, pointing to the begin of direct and indirect regeneration (Table 2, Figure 1).

Regarding explants subjected to various treatments, responses after four weeks of observations are shown in Table 2 and illustrated in Figure 2. The results showed that the concentration of 172 mM NaCl was the most indicated for the selective phase, since it regarded the saline minimum dosage capable of inhibiting regeneration of explants.

Table 2. Results obtained regarding the *in vitro* regeneration potential under saline conditions, as well as the number of necrotic and contaminated explants and their percentage (within parenthesis).

Salt Concent-ration	# explants (100%)		Contaminated explants		Necrotic Explants		Intumesced explants		Indirectly regenerated explants		Directly regenerated explants	
	Cot[1]	Emb[2]	Cot[1]	Emb[2]	Cot[1]	Emb[2]	Cot[1]	Emb[2]	Cot[1]	Emb[2]	Cot[1]	Emb[2]
0 mM	20	10	4 (20)	2 (20)	2 (10)	0	0	0	13 (65)	7 (70)	1 (5)	1 (10)
172 mM	20	10	8 (40)	4 (40)	12 (60)	2 (20)	0	4 (40)	0	0	0	0
258 mM	20	10	8 (40)	4 (40)	12 (60)	6 (60)	0	0	0	0	0	0
344 mM	20	10	2 (10)	1 (10)	18 (90)	9 (90)	0	0	0	0	0	0

Abbreviations: [1]Cotyledons; [2]Embryonic axes.

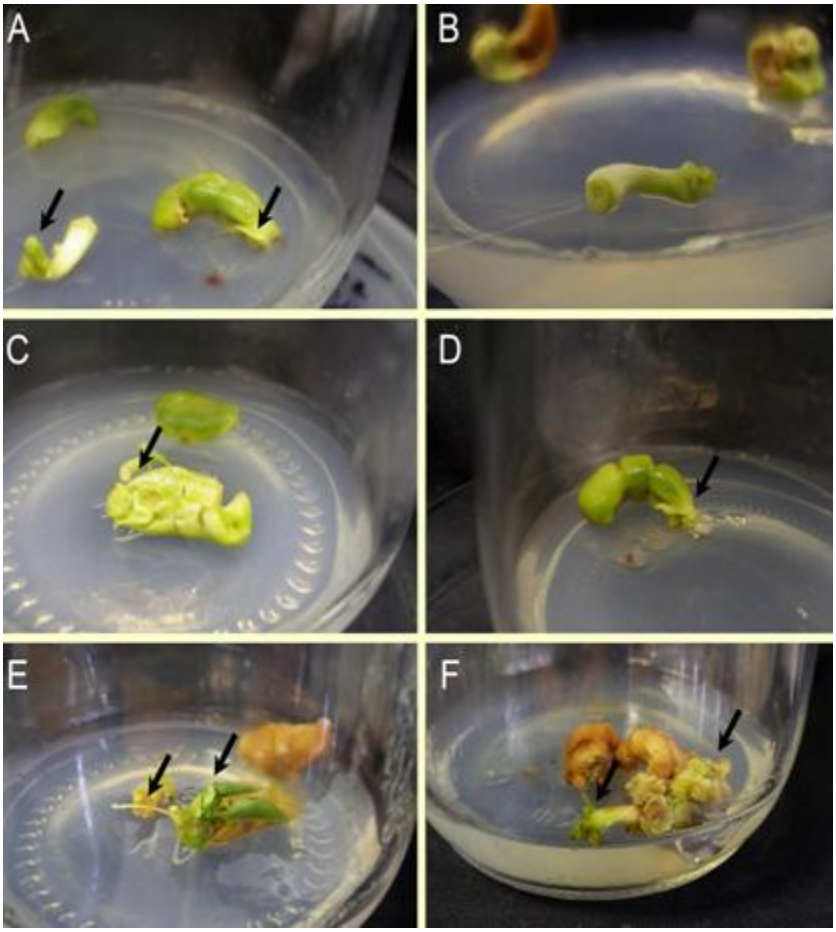

Figure 1. Response of both tissues inoculated (cotyledons and embryo axes) in culture medium without salinity (0% NaCl, negative control). **A.** Greenish tissues indicating the start of regeneration (arrows). **B.** Swollen embryonic axes. **C** and **D.** Cotyledon showing early callus formation (arrows). **E.** Response of the same material as seen in C four weeks after inoculation, indicating the beginning of direct regeneration of a cotyledon, with direct formation of roots (arrows). **F.** Embryonic axes after four weeks, showing the beginning of direct regeneration and callus formation in both tissues (arrows).

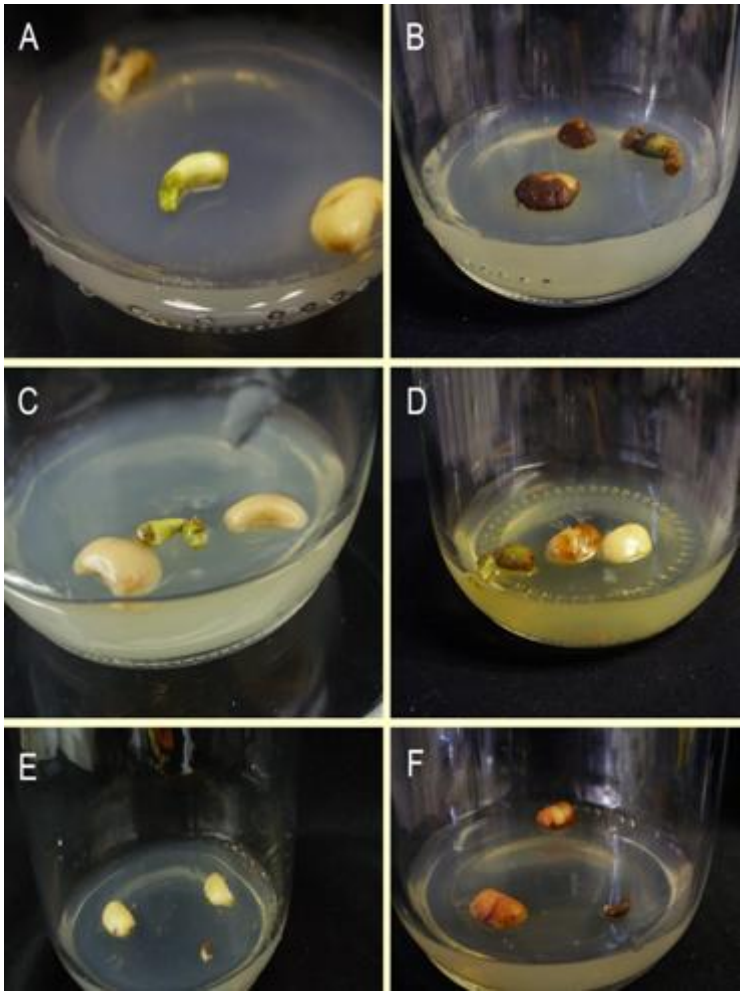

Figure 2. Responses in both tissues inoculated (cotyledons and embryo axes) to different NaCl concentrations during the first four weeks after the experiment. **A.** Tissues inoculated at medium with 1% salt, showing swollen axes and no response in cotyledon. **B.** Also in 1% salt, all tissues necrotic after four weeks of inoculation. **C.** Medium with 1.5% NaCl exhibiting greenish axes and cotyledons with no response. **D.** Tissue with necrosis first signs after four weeks of inoculation in medium with 1.5% NaCl. **E.** Absence of regenerative response in both tissues in culture medium with 2% salt. **F.** Necrotic tissues after four weeks in medium with 2% salt.

To evaluate tissue responses to gamma radiation in relation to the salinity tolerance were considered that the optimum dose for obtaining salinity tolerant mutants would be the one sufficient to induce mutations without severe inhibition of the development, since it would not be possible to distinguish whether the lethality would have been caused by salinity or by high dose of radiation. Therefore we

considered the three doses chosen for this purpose (100, 150 and 200 Gy) and negative control (0 Gy) which were evaluated by counting the different phenotypes of both types of tissues after four weeks, lying on their respective responses. Results are presented in Table 3 for both explants types cultivated in the presence and absence of salt.

Table 3. Main responses in number and percentage (within parenthesis) of explants of both tissues (cotyledons and embryogenic axes) in relation to the radiation dosage in the presence and absence of salt.

Treatment	Radiation in Gy	# explants (100%)		Contaminated explants		Directly regenerated explants		Indirectly regenerated explants		Directly + Indirectly regenerated explants	
		Cot[1]	Emb[2]	Cot[1]	Emb[2]	Cot[1]	Emb[2]	Cot[1]	Emb[2]	Cot[1]	Emb[2]
Without NaCl	0	200	100	32 (16)	16 (16)	36 (18)	20 (20)	28 (14)	20 (20)	12 (6)	12 (12)
	100	200	100	28 (14)	14 (14)	0	26 (26)	28 (14)	36 (36)	0	04 (04)
	150	200	100	28 (14)	14 (14)	0	26 (26)	08 (04)	14 (14)	08 (04)	16 (16)
	200	200	100	24 (12)	12 (12)	0	14 (14)	12 (06)	14 (14)	0	14 (14)
With NaCl	0	200	100	08 (04)	04 (04)	0	0	0	10 (10)	0	0
	100	200	100	28 (14)	14 (14)	0	04 (04)	0	04 (04)	0	0
	150	200	100	24 (12)	12 (12)	0	0	0	0	0	0
	200	200	100	12 (06)	06 (06)	0	0	0	0	0	0

Abbreviations: [1]Cotyledons; [2]Embryogenic axes.

Simultaneously to the above described experiment using radiation doses, another trial was carried out with lower doses (25 Gy 50 Gy and 75 Gy), but the results were not significantly different from those of the control (0 Gy), leading to the

conclusion that they would have no significant influence on responses to regeneration being therefore disregarded for the next steps.

The explants inoculated on non-selective medium presented direct and/or indirect regeneration, being transferred to selective medium in order to verify the presence of possible spontaneous (somaclonal) mutations caused by *in vitro* manipulation in the case of non-irradiated explants confirming the tolerance condition in the case of irradiated individuals.

Among these, only one embryonic axes of the control material (0 Gy) survived to the selection (Figure 3A), being cloned and transferred to new tubes, in order to validate the possible somaclonal variation. One embryo axes irradiated at 150 Gy, previously inoculated on non-selective medium, formed a callus and was thus transferred to two consecutive culture media with the presence of salt, with reduction of the concentration of this selective agent in subsequent transfers (0.6% and 0.3%) until complete salt absence. In this new condition the explants began to develop leaves (Figure 3B).

Explants derived from selective medium that formed callus (indirect regeneration) or presented swelling were transferred for a new medium aiming their maintenance, however, none explant resisted. Those who initiated any kind of response on selective medium were transferred to new selective medium only for maintenance, observing a progressive mortality until they were totally discarded.

Figures 4A-C illustrate graphically how embryonic axes and cotyledons responded to different radiation doses in relation to the direct and indirect regeneration, as well as to both types simultaneously.

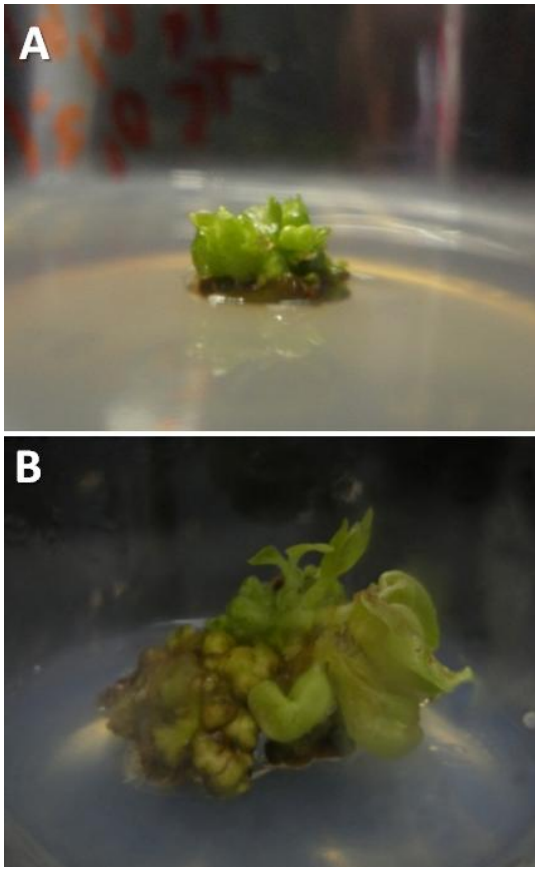

Figure 3. Candidate mutants obtained. **A.** Possible somaclonal mutant obtained from non irradiated material (0 Gy) inoculated in non selective medium and subsequently transferred to selective medium. **B.** Putative solid mutant from explant irradiated at 150 Gy and inoculated in non selective medium and subsequently transferred to selective medium.

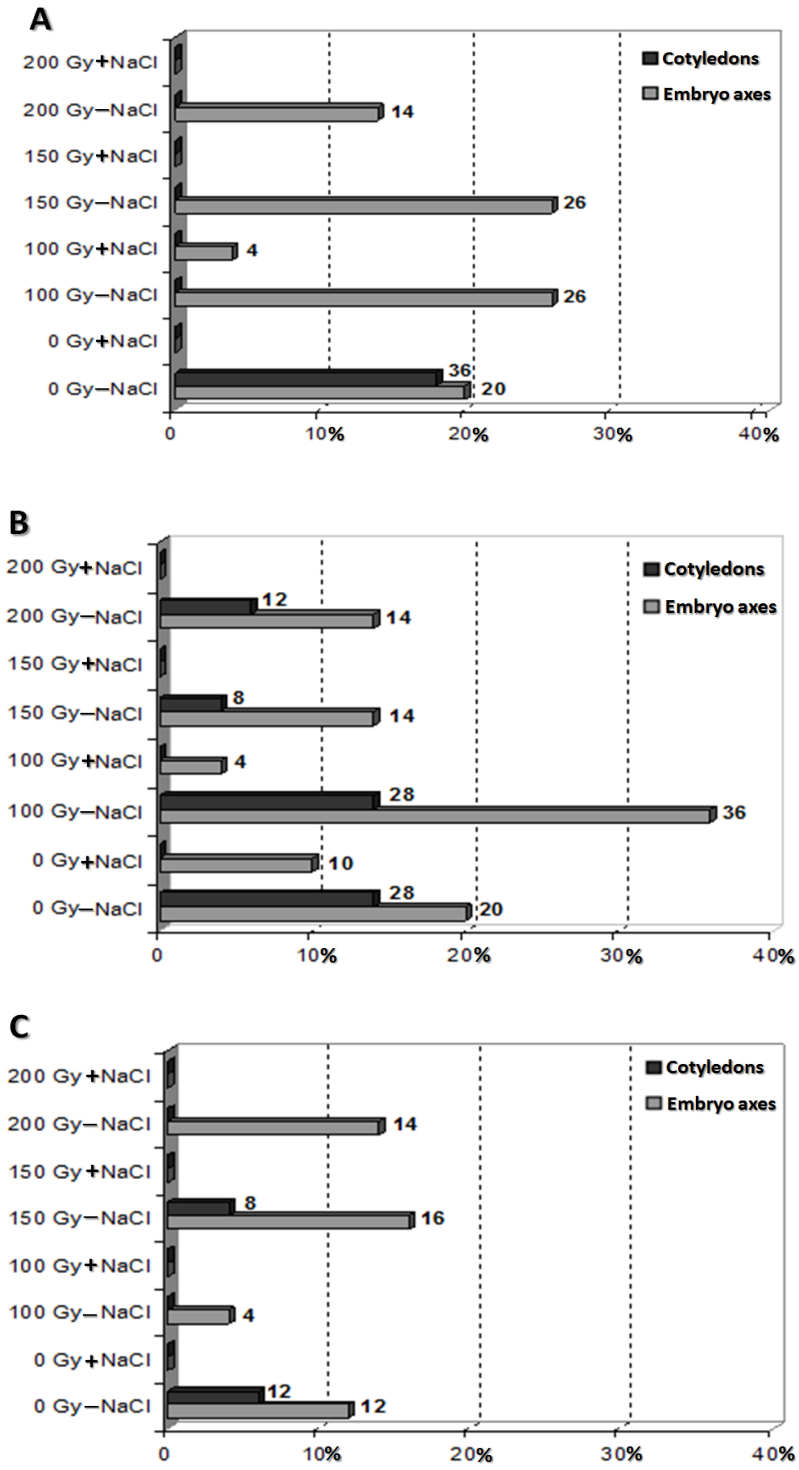

Figure 4. Graphic representation of the percentage of regenerated explants after exposition to different doses of radiation, as well as in the presence or absence of NaCl (concentration 172 mM, 1%). Numbers at the side of each bar represent absolute number of regenerated explants. **A.** Response to *in vitro* regeneration, in relation to direct organogenesis. **B.** Explants that presented indirect organogenesis. **C.** Explants that presented direct organogenesis associated to indirect organogenesis (simultaneously).

4 Discussion

After testing the regenerative potential under saline conditions, treatment 1 (172 mM) proved to be suitable for *in vitro* selection of cultivar BR14-Mulato, being indicated in the present work as the minimal dose able to inhibit tissue regeneration even if in some cases it just started the process.

Hassan et al. (2007) recommended the concentrations of 100 and 150 mM NaCl for *Vigna radiata* and *Solanum lycopersicum*, respectively, for their *in vitro* selection experiments indicating that probably the genotypes exhibit salt sensitivity close to that observed for cowpea cultivar tested here. Germination tests in the presence of different NaCl concentrations have often been used to identify tolerant genotypes including legumes such as alfalfa (Robinson et al., 1986).

The present results indicated that BR14-Mulato cultivar is sensitive to salinity since it showed an inhibition of the regeneration rate in a lower dose (172 mM) than those supported by other cultivars of *V. unguiculata*. Previous studies showed that under NaCl dose considered semi-lethal to many glycophyte plants (200 mM), some cowpea accessions are able to survive for over 20 days without showing any symptoms of injury due to toxicity. Therefore, considering the criterion of survivability, cowpea can be considered tolerant (Silveira et al., 1999), although some cultivars, such as BR14-Mulato, not always provide the same performance as those selected for physiological studies as performed by these authors. Such differences in response to abiotic stresses may be also justified by genotype specific regeneration capability, as highlighted by Zair et al. (2003).

The fact that in previous works some genotypes were classified as tolerant to salinity during germination, flowering or early stage of vegetative growth uncover that the sensitivity of cowpea to salinity changes during its growth and development,

whereas such differences may also be due to environmental factors as temperature, soil composition and other variables as oxygen concentration, light and soil temperature (Murillo-Amador et al., 2006).

The response of explants during *in vitro* selection of irradiated seeds have showed that the dose of 100 and 150 Gy in the absence of NaCl potentiated the direct organogenesis in embryo axes, surpassing even the regeneration rate of the material used as an negative control. At 200 Gy there was a reduction in this percentage, but still presented a satisfactory answer. The same happened with the indirect organogenesis of embryo axes irradiated with 100 Gy and cotyledons irradiated at the same dose, which, in the latter case, was close to that observed in the control treatment. A similar response was reported by Gazzaneo (2007) in a study using the cowpea cultivar IPA 206, which presented a direct organogenesis rate above 60%, differing from the present study regarding the regenerated tissue, since such effects are presented in cotyledons. The cited author also highlighted the effect of different doses of gamma radiation in the frequency of indirect organogenesis in embryonic axes with lower regeneration in tissues subjected to higher dosages.

The results obtained here demonstrate the deleterious effects of high doses of gamma rays, indicating a lower efficiency in obtaining mutants for breeding purposes in higher doses due to explants necrosis. High doses of radiation have been used in the sterilization of plants for storage and consumption in food, while lower doses have been applied on the induction of mutation in seeds, whereas the doses vary, on average, between 60 and 700 Gy for species such as wheat, corn and beans (Ahloowalia and Maluszynski, 2001).

In the scope of *in vitro* mutagenesis studies in cassava (*Manihot esculenta*) Joseph et al. (2004) reported that no tissue survived after exposure to gamma radiation at doses greater than 100 Gy. Particularly, the dose of 50 Gy was able to generate enough variability for the selection of mutants. In a study on the effects of gamma radiation in cultivars of *V. radiata*, Yaqoob and Rashid (2001) also evaluated several characteristics in doses ranging from 100 to 500 Gy, noting that the effects of radiation on the cultivars significantly increased variability in all traits, except for plant height. Furthermore, different cultivars had different responses to different dosages with mutation rates for different genotype-specific characteristics.

In this work, during the *in vitro* selection, genetic variability was detected, indicating somaclonal variation in a non-irradiated individual inoculated into selective medium. Beside the systems of chemical and physical mutagenesis, somaclonal variation can be considered as a third mutagenesis type, also called Biological Mutagenesis (Amano, 2006). Chatterjee and Das Gupta (1998), for example, detected after cytogenetic analysis of rice (*Oryza sativa*) that spontaneous mutations and chromosomal duplications occurred during callus culture, confirming such occurrences, as here proposed. Somaclonal variation is found not only in asexually propagated species, but also in plants sexually propagated including selfing. Although most of the examples of this mutation type regard polyploids, such changes may be also observed in diploid species. The spectrum of properties affected, therefore, can be diverse and the frequency of variants is comparatively high (Larkin and Scowcroft, 1981).

Another part of the results showed that the individual irradiated at 150 Gy and regenerated surviving the selection pressure may likely be a solid mutant, even if the explant initially showed necrotic and non-necrotic spots at the same time,

characteristic indicative of chimerism. The mutants are considered solids because they have all the cells of a histological layer with the same mutant genotype. The mutation comprises a unicellular event and, when multicellular structures are treated with mutagens, if the mutation is induced, there exist in the same plant two or more somatic tissues genetically distinct, a phenomenon denoted as chimerism. Mutants solids can be obtained by reducing the formation of chimeras, thus increasing the frequency of mutation, even in the case of embryogenic callus (Tulmann Neto et al. 1998).

The isolation of whole mutants from chimeras is possible through conventional techniques when organs of the plants are mutated, being difficult to recover when only tiny sections of the plant carry the mutation. However, Datta et al. (2005) succeeded to obtain plants presenting mutated flowers with the same shape and color, indicating no formation of chimeras from mutations induced gamma rays via meristematic tissues. This response was possible due to the fact that mutated cells can regenerate directly through tissue culture *in vitro* leading to the development of a solid mutant (Yamaguchi et al., 2003).

Conclusions

The application of the here tested protocols, allowed some conclusions as follows:

- A biotechnological application of high demand is the 'in vitro mutagenesis'. This technique has allowed an increased genetic variability of the species using very low doses of radiation, which are able to induce punctual mutations (substitutions, insertions or deletions). The practical applicability of the

technique can be found in the mutant bank noted by FAO/IAEA (Program for Nuclear Techniques in Food and Agriculture, 2013).

- The dose of 1% (172 mM) NaCl was the most indicated for the regeneration inhibition, among the tested ones, therefore not impairing growth.
- Gamma ray intensities of 100 and 150 Gy seem to potentiate the regenerative responses in cultivar BR14-Mulato, whereas the dose of 200 Gy presented more deleterious effects.
- Embryo axes responded better to gamma ray exposition and regeneration than cotyledons in BR14-Mulato and probably also in other accessions.
- Even considering that few explants were tested in each approach, one mutant and one somaclonal variant regenerated and survived the selection steps, indicating the potential of the proposed protocol for mutation induction in cowpea, especially considering a higher number of exposed explants.

Considering the promising results observed in other crops, in the present study we sought to establish a protocol to induce mutations associated to salt tolerance in cowpea using a non-tolerant cultivar, establishing not only the minimum dosages for *in vitro* selection pressure under salinity, but also the affordable levels of exposure to gamma rays, and the responses from different explants (cotyledons and embryonic axes) in different steps of the trials. These are the first results of its kind not only in cowpea but in the genus *Vigna* so far, indicating that a reproduction of the protocol may bring a significantly higher number of mutant candidates, useful for lab and field trials.

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Anexo I

Instruções para autores

Transgenic Research

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Transgenic Research

Associated with the International Society for Transgenic Technologies (ISTT)

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Journal no. 11248

Instructions for Authors

Instructions for Authors

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Use italics for emphasis.

Use the automatic page numbering function to number the pages.

Do not use field functions.

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Use the table function, not spreadsheets, to make tables.

Use the equation editor or MathType for equations.

Save your file in docx format (Word 2007 or higher) or doc format (older Word versions).

Word template (zip, 154 kB)

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Footnotes can be used to give additional information, which may include the citation of a reference included in the reference list. They should not consist solely of a reference citation, and they should never include the bibliographic details of a reference. They should also not contain any figures or tables.

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SCIENTIFIC STYLE

Nomenclature: Insofar as possible, authors should use systematic names similar to those used by Chemical Abstract Service or IUPAC.

REFERENCES

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Reference list entries should be alphabetized by the last names of the first author of each work.

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Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. doi:10.1007/s001090000086

Book

South J, Blass B (2001) The future of modern genomics. Blackwell, London

Book chapter

Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

Online document

Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

Dissertation

Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

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www.issn.org/2-22661-LTWA-online.php

For authors using EndNote, Springer provides an output style that supports the formatting of in-text

citations and reference list.

EndNote style (zip, 3 kB)

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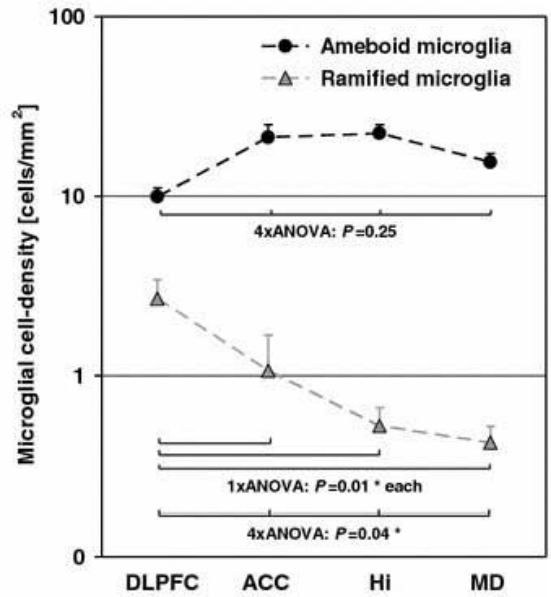

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Do not use faint lines and/or lettering and check that all lines and lettering within the figures are legible at final size.

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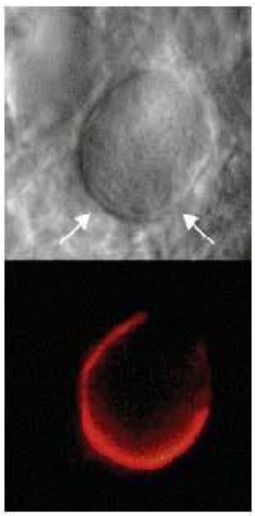
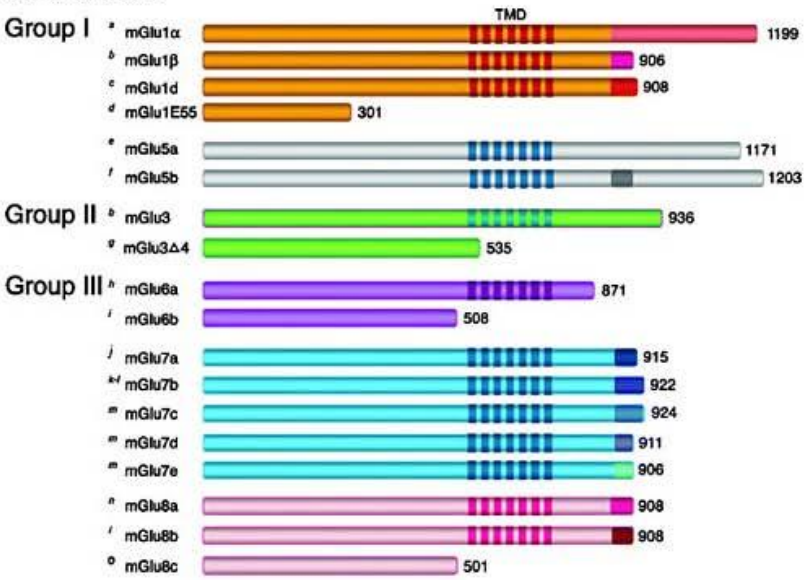


Combination Art



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Anexo II

Instruções para autores

Genetics and Molecular Biology

ISSN 1415-4757 *printed version*
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INSTRUCTIONS TO AUTHORS

Scope and policy

Genetics and Molecular Biology (formerly named Revista Brasileira de Genética/Brazilian Journal of Genetics - ISSN 0100-8455) is published by the Sociedade Brasileira de Genética (Brazilian Society of Genetics).

The Journal considers contributions that present the results of original research in genetics, evolution and related scientific disciplines. Manuscripts presenting methods and applications only, without an analysis of genetic data, will not be considered.

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The official abbreviation for Genetics and Molecular Biology is **Genet. Mol. Biol.**

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b) **The Abstract** must be a single paragraph that does not exceed 200 words and summarizes the main results and conclusions of the study. It should not contain references.

c) **The text** must be as succinct as possible. Text citations: articles should be referred to by authors' surnames and date of publication; citations with two authors must include both names; in citations with three or more authors, name the first author and use et al. List two or more references in the same citation in chronological order, separated by semi-colons. When two or more works in a citation were published in the same year, list them alphabetically by the first author surname. For two or more works by the same author(s) in a citation, list them chronologically, with the years separated by commas. (Example: Freire-Maia et al., 1966a, 1966b, 2000). Only articles that are published or in press should be cited. In the case of personal communications or unpublished results, all contributors must be listed by initials and last name (et al. should not be used). Numbers: In the text, numbers nine or less must be written out except as part of a date, a fraction or decimal, a percentage, or a unit of measurement. Use Arabic numerals for numbers larger than nine. Binomial Names: Latin names of genera, species and infraspecific taxa must be printed in italics; names of orders and families should appear in the Title and also when first mentioned in the text. URLs for programs, data or other sources should be listed in the Internet Resources Section, immediately after the References Section, not in the text.

The text includes the following elements:

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Material (or Subjects) and Methods - Details relevant to the conduct of the study. Statistical methods should be explained at the end of this section.

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