UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS
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Influência do processo de germinação dos grãos de duas cultivares de soja BRS 133 e BRS 258 nos compostos bioativos da farinha integral de soja germinada

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Campinas-SP
2009

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FEA – UNICAMP

Paucar Menacho, Luz Maria
P28i Influência do processo de germinação dos grãos de duas cultivares de soja BRS 133 e BRS 258 nos compostos bioativos da farinha integral de soja germinada / Luz Maria Paucar Menacho. – Campinas, SP: [s.n.], 2009.

Orientador: Yoon Kil Chang
Tese (doutorado) – Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos.

1. Soja. 2. Germinação. 3. Compostos bioativos. 4. ELISA 5. Superfície de resposta. I. Chang, Yoon Kil. II. Universidade Estadual de Campinas. Faculdade de Engenharia de Alimentos. III. Título.

(lpm/fea)

.

Titulo em inglês: Influence of the germination process of the seeds of two soybean cultivars BRS 133 and BRS 258 on bioactive compounds of germinated whole soybean flour.

Palavras-chave em inglês (Keywords): Soybean, Germination, Bioactive compounds, ELISA, Response Surface Methodology

Titulação: Doutor em Tecnologia de Alimentos
Banca examinadora: Yoon Kil Chang
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Maria Teresa Pedrosa Silva Clerici

Data de defesa: 17/02/2009
Programa de Pós-Graduação: Programa em Tecnologia de Alimentos

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A Dios, mi guia, por permitirme conseguir esta victoria en mi vida profesional.

A mis queridos padres Fortunato y Daria Luzmila, por el apoyo incondicional que siempre recibí desde Lima- Perú, cada dia de mi vida en este maravilloso país- Brasil.

A mis queridos hermanos que aún estando separados físicamente, Ana (Lima-Perú) y Miguel (Madrid-España), siempre me apoyaron en seguir adelante con este reto profesional.

A mi querido hermano DAVID ROLANDO PAUCAR MENACHO in memoriam, (03.06.1965 - 21.04.2007)
Murió, cumpliendo con su deber... Dios sabe como me sentí en ese momento, lejos de mi familia...por este acto heroico, David, fue declarado MARTIR de la Policía Nacional del Perú.

Dedico:

A mi amado hijo:

Ricardo Antonio

Hijo mio, tú eres la persona que más apoyo mi decisión de venir a Campinas-BRASIL, para realizar este sueño tan deseado desde hace muchos años...

Infinitas gracias, mi amor, por la paciencia y espera de estos largos 4 años!

AGRADECIMENTOS

A Deus, pela força durante minha permanência no Brasil.

A Dra. Gláucia Maria Pastore, Diretora da FEA, que abriu meus caminhos para realização deste trabalho.

Ao meu orientador, Prof. Dr. Yoon Kil Chang, pela amizade, condução do trabalho de pesquisa e confiança depositada em mim na realização deste trabalho.

À Universidad Nacional del Santa - UNS, no Perú, pela liberação concedida nestes 4 anos, para a realização do meus estudos de doutorado.

Aos meus colegas da Faculdade de Engenharia da UNS, M. Sc. Augusto Castillo Calderon, Saúl Eusebio Lara, Jorge Dominguez Castañeda, Elizalde Carranza Caballero e Vicente Carranza Varas, dos quais recebi apoio incondicional para vir a realizar meus estudos de doutorado.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) através do Programa Estudante Convênio Pós-Graduação (PEC-PG) pela bolsa de doutorado concedida.

À FEA, DTA e UNICAMP, em especial ao Laboratório de Cereais, Raizes e tubérculos DTA/FEA, pelo suporte institucional.

A Dra. Mercedes Concórdia Carrão-Panizzi, pesquisadora da Embrapa Soja, na Área de Melhoramento Genético pela sugestão da escolha das cultivares BRS 133 e BRS 258, para o desenvolvimento desta pesquisa.

Ao Dr. Fernando Matsuura, da Embrapa Transferência de Tecnologia, pela cessão dos grãos de soja.

A Rosa Helena Aguiar, técnica do Laboratorio do Laboratório de Pós-Colheita da Faculdade de Engenharia Agrícola-UNICAMP, pelo auxílio na execução dos processos de germinação e amizade.

Ao Dr. José Marcos Gontijo Mandarino, pesquisador da Embrapa Soja, na área Técnica de Genética e Melhoramento, pelo suporte e apoio nas análises de isoflavonas.

A Dra. Elvira Gonzáles de Mejía, pelo convite para realizar as análises dos compostos bioactivos no laboratório “Food Science and Human Nutrition in University of Illinois at Urbana-Champaign-USA”.

Ao Rodolfo Rohr Neto (SoSoja do Brasil Ltda) e Kenji S. Narumiya (Sun Foods-Brasil), FAEPEX processo 149/08, pelo apoio para minha transferência e suporte econômico durante minha permanência nos Estados Unidos.

Ao Dr. Mark A. Berhow, Membro do Department of Agriculture-USDA, Agricultural Research Service, Peoria, IL, United States, pelo suporte e apoio nas análises de saponinas.

Ao Dr. Jaime Amaya Farfán, professor responsável do Laboratório de Fontes Protéicas do DEPAN, pela amizade e apoio nas análises de aminoácidos.

Ao Dr. Flavio Luis Schmidt, professor responsável do Laboratorio de Frutas e Hortaliças, pela disponibilidade do uso do liofilizador para o desenvolvimento desta pesquisa.

A profa. Dra. Lireny Ap. G. Gonçalves, quem deu muito carinho, dedicação e força durante minha permanência no Brasil, minha familia e eu, viveremos eternamente gratos a você.

A Profa. Dra. Maria Isabel Rodrigues (DEA), pelos esclarecimentos e ensinamentos da parte estatística.

A meus pais, Daria Luzmila e Fortunato, de quem sempre recebi o apoio incondicional para continuar com minha capacitação, vocês são os anjos que cuidaram de meu filho, por isso e por tudo que me ensinaram na vida, minha eterna gratidão.

A meus irmãos Ana e Miguel, pela força recebida durante todo o tempo de minha permanência no Brasil.

A meu filho Ricardo Antonio, pela força para conseguir este sonho tão desejado.

A Maria Teresa Pedrosa Silva Clerici, pelo carinho, amizade e, sobretudo pelos ensinamentos e conselhos recebidos em todo momento no desenvolvimento da tese.

A Patricia Luna Pizarro, professora da Universidade de Jujuy- Argentina, pelo carinho, amizade e ajuda recebida durante sua permanência no laboratório de Cereais.

Ao Anderson de Souza Sant´Ana, pela sua amizade, carinho, companherismo, paciência e dedicação durante a minha permanência no Brasil, amizade que perdurará por toda minha vida.

Ao Matheus Depieri e família, pela amizade, carinho e força recebida em todo momento.

A Luciana Cristina Brigato Fontes e familia, pelo carinho recebido, desde o primeiro dia no curso, minha eterna gratidão.

A Alessandra Silva Coelho, técnica do laboratório de Cereais, DTA, FEA, UNICAMP, pela amizade e ajuda recebida.

A Carla Greghi, técnica do Laboratorio de Fontes Protéicas do DEPAN, FEA, UNICAMP, pela amizade e ajuda recebida.

Aos meus queridos amigos do Laboratorio de Cereais; Márcio, Reinaldo, Noé Leomar, Gabriela, Camila, Eliza, Leandra, Paula e Eveline, pelo companherismo e amizade recebida, minha eterna gratidão.

Aos amigos da pós-graduação do DTA, DCA e do DEA e a todas as pessoas que de formas diversas me apoiaram e contribuíram para a conclusão deste trabalho.

Obrigada!

ÍNDICE GERAL

RESUMO

O consumo de soja tem aumentado consideravelmente nos últimos anos, devido à suas propriedades funcionais com a presença de diversos compostos bioativos como as isoflavonas, das quais as mais importantes formas são a genisteína e a daidzeína que, em determinadas concentrações, trazem benefícios para à saúde dos consumidores. Novos compostos protéicos bioativos estão sendo pesquisados, tanto, como a lunasina, o Inibidor de Bowman-Birk (BBI) e a lectina, e como não protéicos, as saponinas. O objetivo deste trabalho foi estudar a influência dos parámetros do processo de germinação (tempo e temperatura) das cultivares de soja BRS 133 (baixo teor protéico) e BRS 258 (alto teor protéico) desenvolvidas pela EMBRAPA, nos compostos bioativos da farinha integral de soja germinada (FISG). Os efeitos das variações de tempo e temperatura de germinação nos compostos bioativos foram analisados através da Metodologia de Superfície de Resposta, com um delineamento composto central rotacional com duas variáveis independentes: tempo de germinação (x_1) e temperatura de germinação (x_2). O delineamento incluiu onze ensaios: quatro pontos fatoriais, quatro pontos axiais e três repetições no ponto central. A germinação foi realizada em câmara de germinação, entre papéis, e no final dos tempos e temperaturas de germinação segundo o planejamento do experimento as amostras foram congeladas a -30 ° C, e depois liofilizadas. As con centrações de isoflavonas e saponinas foram determinadas por cromatografia líquida de alta eficiência (CLAE) e as concentrações de proteína solúvel, lunasina, Inibidor de Bowman Birk (BBI) e lectina foram determinadas por ELISA (enzyme-linked immunosorbent assay). A identificação do polipeptídeo bioativo lunasina foi determinado por Western Blot e a atividade da lipoxigenase foi determinada por quantificação da banda de lipoxigenase em gel por eletroforese. A caracterização físico-química das duas cultivares de soja brasileira permitiu concluir que, embora a sua composição esteja dentro de uma gama típica de nutrientes da soja, surge um padrão distinto de alguns nutrientes e de compostos bioativos, no que diz respeito ao teor de

proteínas. A cultivar BRS 133 apresentou um baixo teor de proteína e uma alta concentração de isoflavonas totais e, em forma oposta, a cutivar BRS 258 apresentou um alto teor de proteínas e baixa concentração de isoflavonas totais. Os resultados mostraram que, tanto o tempo como as temperaturas de germinação tiveram uma influência significativa sobre a composição e as concentrações de compostos bioativos na farinha de soja germinada. Nesta pesquisa, foram determinadas as faixas ótimas de tempo e temperatura de germinação para obter o maior conteúdo de compostos bioativos (lunasina, isoflavonas e saponinas) e a diminuição de fatores antinutricionais (BBI e lectina). Neste estudo, também foram determinados estes compostos, embora sejam antinutricionais, pois atualmente são considerados bioativos e com benefício à saúde. Na cultivar BRS 133, um tempo de germinação de 42 horas a 25 ℃ resultou em um aumento de 61,66% na concentração de lunasina, uma diminuição de 58,73% na concentração de lectina e uma diminuição de 69,95% na atividade de lipoxigenase. Aumentos significativos na concentração de isoflavonas agliconas (daizeína e genisteína) e na concentração de saponinas totais foram observados com um tempo de germinação de 63 h a uma temperatura de 30℃. Sob estas condições, a concentração de genisteína na FISG comparada com o grão de soja sem germinar, apresentou um aumento de 212,29% neste flavonóide bioativo. Na cultivar BRS 258, o processo germinativo resultou numa redução do BBI, da lectina e da atividade de lipoxigenase. Um baixo tempo de germinação, 12 h (-1), a 25 ℃ (0) resultou em maior concentração de lunasin a. Um aumento no tempo de germinação de 12 h (-1) para 72 h (+1), a 25 ℃ res ultou em um aumento de 31,9% no teor de proteína solúvel, um decréscimo de 27,0% na concentração BBI, e uma diminuição de 72,6% na concentração de lectina. Nesta cultivar, aumentos significativos na concentração de isoflavonas agliconas (daizeína e genisteína) e nas saponinas totais foram observados com um tempo de germinação de 63 h a 30 ℃.

Palavras-chave: soja, BRS 133, BRS 258, germinação, lunasina, Inibidor de Bowman-Birk (BBI), lectina, saponinas, isoflavona.

ABSTRACT

The consumption of soybean has increased considerably in recent years due to its functional properties, with the presence of many bioactive compounds such as isoflavones, of which the most important forms are genistein and daidzein, which, in determined concentrations, can provide health benefits to the consumer. New bioactive protein compounds are also being studied, such as lunasin, the Bowman-Birk inhibitor (BBI), lectin and non-protein bioactive compounds such as saponins. The objective of the present work was to study the influence of the parameters of the process of germination (time and temperature) of the soybean cultivars BRS 133 (low protein) and BRS 258 (high protein), both developed by EMBRAPA, on the bioactive compounds in the whole flour obtained from the germinated soybean (GSWF). Response surface methodology was used to analyze the effects of variations in germination time and temperature on the bioactive compounds using a central composite rotational design with two independent variables: germination time (x_1) and germination temperature (x_2). The design included eleven trials: four factorial points, four axial points and three repetitions at the central point. Germination was carried out between papers in a germination chamber, and at the end of the times and temperatures determined by the experimental design, the samples were frozen at -30ºC and subsequently freeze dried. The concentrations of isoflavones and saponins were determined by high performance liquid chromatography (HPLC), and the concentrations of soluble protein, lunasin, Bowman-Birk inhibitor and lectin by ELISA (enzyme-linked immunosorbent assay). The identification of the bioactive polypeptide lunasin was determined by the Western Blot assay and lipoxygenase activity by quantification of the band obtained in gel electrophoresis. The physicochemical characterization of the two Brazilian soybean cultivars allowed to conclude that, although their compositions were within the typical ranges for soybean nutrients, there was a distinct pattern for some nutrients and bioactive compounds with respect to the protein contents. The cultivar 133 presented a low protein content

and high concentration of total isoflavones, whereas the cultivar BRS 258 presented a high protein content and low concentration of total isoflavones. The results showed that both the germination time and temperature had a significant influence on the composition and concentrations of bioactive compounds in the germinated soybean whole flour (GSWF). The optimum germination time and temperature ranges to obtain maximum contents of the bioactive compounds (lunasin, isoflavones and saponins) and maximum decrease in the anti-nutritional factors (BBI and lectin), were determined in this study, although currently these anti-nutritional factors are considered bioactive and of benefit to health. Using the cultivar BRS 133, a germination time of 42 hours at 25ºC resulted in an increase of 61.66% in lunasin concentration, a decrease of 58.73% in lectin concentration and a decrease of 69.95% in lipoxygenase activity. A significant increase in the concentrations of the aglycone isoflavones (daizein and genistein) and total concentration of saponins was observed with a germination time of 63 h at a temperature of 30ºC. In these conditions, genistein concentration in GSWF, as compared to the non-germinated soybean, resulted in an increase of 212.29% of this bioactive flavonoid. With the cultivar BRS 258, the germination process resulted in a reduction in BBI, lectin and lipoxygenase activity. Low germination times of 12 h (-1) and temperatures 25 ºC (0) resulted in a greater concentration of lunasin. An increase in the germination time from 12 h (-1) to 72 h (+1) at 25 ºC resulted in a 31.9% increase in soluble protein, a 27.0% decrease in the concentration of BBI and a 72.6% decrease in lectin concentration. With this cultivar, a significant increase in the concentration of the aglycone isoflavones (daizein and genistein) and total saponins was also observed with a germination time of 63 h and temperature of 30 ºC.

Keywords: soybean, BRS 133, BRS 258, germination, lunasin, Bowman-Birk Inhibitor (BBI), lectin, saponins, isoflavone.

Introdução geral

O interesse e a busca do consumidor por alimentos mais saudáveis propiciam um rápido crescimento do segmento da indústria de alimentos que visa contribuir para o alcance de uma dieta de melhor qualidade. Devido à imagem negativa do uso de medicamentos, e incertezas associadas à eficiência dos suplementos, a procura por alimentos de efeito benéfico à saúde tem se tornado bastante popular.

A soja e seus derivados têm sido utilizados há séculos nos países orientais como alimento básico da dieta daquelas populações, além de serem usados como ingredientes para produtos industrializados no ocidente. Pesquisas revelaram que a incidência e mortalidade causada pelo câncer de mama em mulheres ocidentais têm sido consideravelmente mais elevadas que na Ásia, onde a soja tem importante papel na dieta. Estudos epidemiológicos demonstraram que, além do câncer de mama e doenças cardiovasculares, a osteoporose, câncer de próstata e os sintomas da menopausa são raros nas sociedades asiáticas, demonstrando, assim, que a soja tem papel preventivo e terapêutico na saúde do indivíduo.

A soja, no Brasil, é um dos produtos agrícolas de grande importância econômica, com sua produção atingindo volumes recordes nos últimos anos. Em 2007, o país foi o segundo maior produtor mundial de soja, com 58 milhões de toneladas, o que corresponde a 27% da safra mundial (FAO, 2008), com projeções de aumento até o 2015 (MINISTERIO DE AGRICULTURA – AGE, 2005).

Estudos demonstram que o tratamento térmico, a germinação, a fermentação, e a hidrólise química ou enzimática, promovem alterações nos compostos químicos, alterando os isômeros das isoflavonas, hidrolisando as proteínas e reduzindo os fatores antinutricionais (MOLTENI, *et al.,* 1995; KIM *et*

al., 1998; ZHU *et al*., 2005)

O processo de germinação proporciona aumento no valor nutritivo, pela melhoria da digestibilidade protéica e pelo aumento do valor do quociente de eficiência protéica (QEP), redução dos fatores antinutricionais nas leguminosas, tais como inibidores proteolíticos e lectinas, provocando a hidrólise de oligossacarídeos (rafinose e estaquiose) presentes na soja, os quais são causadores de flatulência. A germinação também proporciona um aumento do teor de metionina, aminoácido limitante da proteína de soja (BARCELOS *et al*., 2002).

Sendo assim, o objetivo geral deste trabalho foi estudar a influência do processo de germinação das variedades de soja BRS 133 e BRS 258 nos compostos bioativos da farinha integral de soja germinada (FISG).

Para alcanzar este objetivo colocou-se os seguintes objetivos específicos:

- Determinar as propriedades físico-químicas da farinha integral de soja (FIS) das variedades BRS 133 e BRS 258, desenvolvidas pela área Técnica de Genética e Melhoramento da EMBRAPA-Brasil.
- Desenvolver o processo de germinação das variedades de soja BRS 133 e BRS 258 em condições de laboratório com diferentes tempos e temperaturas de germinação segundo o planejamento estatístico (DCCR) do tipo composto central rotacional 2^2.
- Obter uma farinha integral de soja germinada (FISG) a partir das sementes de soja germinadas visando à preservação dos seus compostos bioativos.
- Determinar e quantificar os compostos bioativos na FIS e na FISG das variedades de soja BRS 258 e BRS 133.
- Determinar as faixas ótimas de tempo e temperatura de germinação nas duas cultivares de soja, visando ao aumento dos compostos bioativos (lunasina, isoflavonas agliconas e saponinas) e diminuição dos compostos antinutricionais (BBI e lectina).

Capítulo 1: Revisão Bibliográfica

1. Alimentos funcionais

As plantas são fontes de diferentes compostos químicos bioativos de grande importância para a medicina, os quais são objetos de inúmeras investigações científicas e uso empírico por pessoas da zona rural e também da zona urbana. Muitas destas plantas são amplamente consumidas na dieta humana, podendo ser benéficos à saúde (MACIEL *et al*., 2002).

O termo “alimentos funcionais” foi inicialmente proposto no Japão, em meados dos anos 80, sendo na época o único país que formulou um processo de regulação específico para os alimentos funcionais. Conhecidos como alimentos de uso específico para a saúde “Food for a Specific Health Use - FOSHU, estes alimentos são qualificados e trazem um selo de aprovação do Ministério de Saúde e Previdência Social Japonês (CÂNDIDO, 2002).

No Brasil, a regulamentação é feita pela ANVISA, que em 1999 publicou duas resoluções relacionadas aos alimentos funcionais; Resolução n° 18, de 30/04/1999 (republicada em 03/12/1999), a qual aprova o regulamento técnico que estabelece as diretrizes básicas para análise e comprovação de propriedades funcionais e/ou de saúde alegadas em rotulagem de alimentos (BRASIL, 1999) e a Resolução n° 19, de 30/04/1999 (republicada em 10/1 2/1999) que aprova o Regulamento Técnico de procedimentos para registro de alimento com alegação de propriedades funcionais e/ou de saúde em sua rotulagem (BRASIL, 1999).

Essas resoluções fazem distinção entre alegações de propriedade funcional e alegação de propriedades de saúde, como segue:

Alegação de propriedade funcional: é aquela relativa ao papel metabólico ou fisiológico que o nutriente ou não nutriente tem no crescimento, desenvolvimento, manutenção e outras funções normais do organismo humano.

Alegação de propriedade de saúde: é aquela que afirma, sugere ou implica a existência da relação entre o alimento ou ingrediente com doença ou condição relacionada à saúde.

Uma definição abrangente de alimento funcional poderia ser "qualquer alimento natural ou formulado pelo homem, que contenha uma ou mais substâncias, classificadas como nutrientes ou não-nutrientes, capazes de atuar no metabolismo e na fisiologia humana, promovendo efeitos benéficos à saúde, retardando, inclusive, processos patológicos que conduzem a doenças crônicas e/ou degenerativas, melhorando a qualidade e a expectativa de vida das pessoas" (PACHECO e SGARBIERI, 2001).

Dentre os componentes dos alimentos com funcionalidade fisiológica podem-se citar, entre os nutrientes: as fibras; os ácidos graxos poliinsaturados da família ômega-3, como o EPA (ácido eicosapentaenóico) e o DHA (ácido docosaexaenóico); minerais essenciais; proteínas e peptídeos; e, entre os não-nutrientes: os oligossacarídeos; os flavonóides, como as isoflavonas da soja; os carotenóides; os fitoesteróis e compostos fenólicos (SGARBIERI e PACHECO, 2001; LAJOLO, 2001).

A legislação americana define um alimento funcional como *suplemento dietético, alimento ou alimento medicinal que possui benefícios à saúde e é seguro para consumo humano em qualidade e freqüência requeridas para se alcançar a propriedade sugerida ao produto.* Muitos alimentos ou componentes de alimentos são ditos nutracêuticos e têm sido adicionados a alimentos industrializados como o suco de laranja enriquecido com cálcio, que tem propriedade de prevenir a osteoporose (HENRY, 1999).

A Tabela 1 mostra a lista dos doze alimentos ou componentes relacionados à saúde humana aprovados pela FDA (Tabela 1).

Tabela 1. Alimentos e componentes relacionados a algum dano ou benefício à saúde.

Alimentos/Componentes	Relação com a saúde
Cálcio	Previne osteoporose
Dieta rica em lipídios saturados	Pode causar câncer
Sódio	Pode causar hipertensão
Dieta rica em gordura saturada e colesterol	Pode causar doenças coronárias
Grãos, frutas e vegetais ricos em fibras	Previnem câncer
Grãos, frutas e vegetais ricos em fibras solúveis	Previnem doenças coronárias
Frutas e vegetais	Previnem câncer
Folatos	Protege contra defeitos no tubo neural
Polióis	Protege contra cáries dentárias
Fibras solúveis de aveia ou vagem	Protegem contra doenças coronárias
Proteína de soja	Previnem contra doenças coronárias
Grãos integrais	Previnem certos tipos de câncer, incluindo de intestino, cólon, esôfago e estômago

Fonte: HENRY, 1999.

Baseado nas evidências da soja em ajudar a prevenir os riscos de doenças cardíacas, o FDA (Food and Drug Administration) aprovou a indicação no rótulo de que os produtos à base de soja trazem benefícios à saúde. Para isso, tais produtos necessitam apresentar um teor de 6,25 g de proteína de soja por porção consumida, além de conter baixos teores de gordura saturada e colesterol. Esse valor equivale a ¼ (representando quatro refeições diárias) dos 25 g de proteína de soja considerados necessários para se promover à diminuição dos níveis de lipídios no sangue e ajudar a prevenir doenças cardíacas (UNITED STATES, 2002).

Diversos estudos realizados comprovaram que o consumo de soja reduz a quantidade de lipídios no sangue, que estão associados a redução dos riscos de doenças cardiovasculares (KERCKHOFFS *et al*., 2002; TIKKANEN & ADLERCREUTZ, 2000; JENKINS *et al*., 2000; SETCHELL, 1998; ANDERSON *et al*., 1995; CARROLL, 1991) e ajuda na prevenção do câncer (MESSINA & MESSINA, 1991).

2. Soja

Soja (*Glycine max* L. Merril) é uma leguminosa originária da China e difundida no Ocidente, principalmente por constituir-se em uma importante fonte de óleo para o consumo humano e ração animal. Apresenta-se como importante fonte de proteínas, embora seja ainda subaproveitada na dieta humana e em produtos industrializados. Devido à grande quantidade de proteína por área plantada que fornece, é chamada de "jóia amarela", "grande tesouro", "proteína milagrosa da natureza" e "carne do campo". Também é vista hoje como uma das principais armas no combate contra a fome e a desnutrição no mundo (Figura 1).

2.1 Produção mundial de soja

Segundo a FAO (2008), o Brasil ocupa o segundo lugar em produção mundial de soja, respondendo por 27% do total de soja produzida no mundo, com aproximadamente 58,2 milhões de toneladas no ano de 2007, ficando atrás apenas dos EUA, com 84 milhões de toneladas. Outros grandes produtores mundiais são Argentina, China, Índia, Paraguai e Canadá. Na tabela 2, observa-se a evolução da produção de soja entre os países líderes nos últimos anos. O Brasil apresenta uma tendência acentuada de crescimento na produção de soja nos últimos anos. Percebe-se que a produção da Argentina acompanha o perfil da produção brasileira, enquanto os EUA mostram uma queda seguida de uma recuperação da produção no último ano. Por outro lado, a China mantém uma produção estável, cabendo-lhe o papel de grande importador mundial deste grão.

Classificação Cientifica

Reino : *Plantae*

Divisão : *Magnoliophyta*

Classe : *Magnoliopsida*

Ordem : *Fabales*

Família: *Fabaceae*

Subfamília: *Faboideae*

Gênero: *Glycine*

Espécie: *G. max.*

Nome binomial

Glycine max (L.) Merril

Figura 1. Classificação científica da soja

TABELA 2. Maiores produtores mundiais de soja (em milhões de toneladas).

País		Anos										
		1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007
1º	***Estados Unidos***	73,2	74,6	72,2	75,1	78,7	74,8	65,8	85,7	83,9	87,6	70,7
2º	***Brasil***	26,4	31,3	31,0	32,7	37,9	42,1	51,5	49,2	52,7	52,3	58,2
3º	***Argentina***	11,0	18,7	20,0	20,2	26,9	30,0	34,8	32,0	38,3	40,4	45,5
4º	***China***	14,7	15,2	14,2	15,4	15,4	16,5	16,5	17,7	17,4	15,5	15,6
5º	***Índia***	6,5	7,1	7,1	5,3	6,0	4,6	6,8	7,0	6,3	8,2	9,4
6º	***Paraguai***	2,7	2,9	3,1	3,0	3,5	3,3	4,4	3,8	3,5	3,8	3,9
7º	***Canadá***	2,7	2,7	2,8	2,7	1,6	2,3	2,3	2,9	2,9	3,5	2,8
	Outros	7,2	7,6	7,4	7,0	6,8	7,3	7,1	8,1	9,3	10,1	10,0

Fonte: FAO, 2008.

2.2 Composição centesimal do grão de soja

Os grãos de soja constituem uma rica fonte de proteínas e óleo com teores médios de 38% e 19%, respectivamente segundo Liu (1997), como pode ser observado na Tabela 3. Sua composição química, com base em 100 g de amostra seca, constitui-se de 40 g de proteínas, 30 g de glicídeos, 20 g de lipideos, 226 mg de cálcio, 546 mg de fósforo e 8,8 mg de ferro (WOLF & COWAN, 1971; SGARBIERI *et al*., 1981). O óleo e grande parte da proteína dos grãos de soja se encontram em corpúsculos especiais contidos nas células cotiledôneas. Os corpúsculos contendo o óleo medem de 0,2 a 0,3 μm de diâmetro e são chamados esferossomos. As proteínas são armazenadas em corpúsculos maiores, 2 a 20 μm de diâmetro, denominados grãos de aleurona ou corpúsculos protéicos. Os corpúsculos protéicos são envoltos por uma membrana fosfolipídica que é estável na presença de éter dietílico e hexano. Podem ser isolados por centrifugação em gradiente de densidade e, normalmente, apresentam elevado conteúdo protéico (SGARBIERI, 1996).

Dos glicídeos totais, 4 a 5% são sacarose, 1 a 2% rafinose, e 3,5 a 4,5% estaquiose. Embora todos os açúcares sejam fermentados por microrganismos, os oligossacarídeos rafinose e estaquiose têm um importante papel bifidogênico, ainda que não sejam digeridos pelos seres humanos e outros animais monogástricos. Estes açúcares são responsáveis pelo fenômeno da flatulência nos seres humanos e em animais, e quando presentes nas rações, acarretam uma perda da eficiência alimentar (MORAES, 2002).

Tabela 3. Composição centesimal do grão de soja.

Componente	% em base seca
Proteína	40,0
Lipídios	20,0
Carboidratos	35,0
Cinzas	5,0

Fonte: Liu, 1997.

Os grãos de soja contêm menos que 1% de amido, 5% de cinzas e 4,5% de fibra bruta. Mais da metade da fibra bruta existente na soja é considerada como fibra alimentar, cujo desempenho fisiológico é necessário para um melhor aproveitamento dos nutrientes pelos seres humanos. A importância da fibra alimentar na dieta tem recebido muita atenção em diversas partes do mundo. Embora o papel desempenhado pelas fibras na redução de incidência de câncer de cólon e de doenças cardíacas não esteja bem elucidado, os benefícios potenciais para a saúde gerados pelo aumento do teor de fibras na dieta não devem ser desprezados. A casca da soja contém por volta de 87% de fibra bruta, sendo formada por celulose, hemicelulose, lignina e ácidos urônicos (ERICKSON, 1995).

O cálcio e o fósforo são os minerais de maior significância na soja. O principal interesse nutricional do conteúdo de cálcio na soja relaciona-se com a comparação que se faz entre o “leite” de soja e o de vaca. O teor de cálcio nestes dois tipos de leite é semelhante; o leite de vaca contém 0,11%, contra 0,08% do “leite” de soja, quando preparado, este último, da maneira tradicional. A biodisponibilidade de cálcio no “leite” de soja (22,2%) é aproximadamente 90% da biodisponibilidade deste mineral no leite de vaca (29,1%). Do total de cálcio contido no grão de soja cozido (0,16-0,47%), apenas 10% é efetivamente utilizado pelo homem (MORALES, 1985).

Em relação ao fósforo, os compostos que contribuem com este mineral na soja são: fósforo inorgânico, fitina, diferentes fosfolipídios e ácidos nucléicos. Contudo, as principais fontes na soja são as fitinas ou ácido fítico, que contribui com 50 a 70% do total de fósforo. Os fosfolipídios, substâncias semelhantes às gorduras que possuem nitrogênio e fósforo na sua molécula, representam a segunda grande fonte de fósforo na soja, contribuindo com aproximadamente 15% do total (MORALES, 1985).

A proteína da soja é pobre em aminoácidos sulfurados, sendo que a metionina é o aminoácido limitante, seguido do triptofano, cisteína e treonina (Tabela 4). Entretanto, é uma proteína rica em lisina, aminoácido deficiente na maioria dos cereais. Por isso a combinação de proteínas de leguminosas, como no caso a soja, e de cereais, como o milho, é valiosa, já que são complementares em relação à metionina e lisina.

Tabela 4. Composição dos aminoácidos da soja comparada à necessidade humana e de ratos.

Aminoácido	Presente na soja (mg/g de proteína)**	Requerimento WHO/FAO/UNU* necessário (mg/g)			
		Crianças*		Adultos*	Ratos**
Essenciais		*1-2 anos*	*3-10 anos*		
Histidina	34	-	-	15	25
Isoleucina	52	-	-	30	46
Leucina	82	-	-	59	62
Lisina	68	52	48	45	75
Metionina Cisteína	11 25	26	24	24	50
Fenilalanina Tirosina	56 42	-	-	38	67
Treonina	42	27	25	23	42
Triptofano	13	74	66	6	12
Valina	54	-	-	39	50
TOTAL	479	179	163	279	429
Não-essenciais					
Alanina	40	-	-	-	-
Arginina	77	-	-	-	50
Ácido aspártico	69	-	-	-	-
Ácido glutâmico	190	-	-	-	-
Glicina	37	-	-	-	-
4-Hidroxiprolina	1,4	-	-	-	-
Prolina	53	-	-	-	-
Serina	54	-	-	-	-

Fonte : * WHO/FAO/UNU, 2007.

** Liu, 1997.

Apesar dos aminoácidos sulfurados (metionina e cisteína) serem considerados limitantes no caso de leguminosas, na soja, no entanto, o teor desses aminoácidos é suficientemente alto e responde às necessidades da dieta

humana quando se considera uma quantidade de proteína adequada. A qualidade da proteína da soja é subvalorizada porque o método tradicional de avaliação, a taxa de eficiência da proteína (PER), baseia-se no crescimento de cobaias, principalmente ratos, os quais possuem uma necessidade de metionina aproximadamente 50% maior que a de humanos. Quando se utiliza o novo método de avaliação da qualidade de proteína proposto pela OMS e pela FDA americana, conhecido como PDCAAS (*Protein Digestibility Corrected Amino Acid Score*), que compara o padrão de aminoácidos de uma proteína com o necessário na dieta, associando a isso um fator de correção para a digestibilidade, a proteína da soja consegue obter valores entre 0,95 e 1, os mais altos possíveis (MESSINA, 1997).

2.3 Cultivar BRS 133

A soja BRS 133 pode ser semeada em solo de baixa a média fertilidade; com alto potencial de rendimento; é excelente em ambiente altamente produtivo para uma excelente ramificação da planta. Deve-se evitar semeadura em solos compactos ou em solos que apresentam problemas de drenagem. Também pode ser indicada para áreas de reforma de canavial. As áreas de adaptação são PR, SP, SC e MS. Tem um teor de proteína de 38,60% e teor de óleo de 18%. A cor de sua flor é branca e a cor do hilo é marrom (EMBRAPA, 2008).

2.4 Cultivar BRS 258

A cultivar Embrapa 258 é originaria da cultivar BR 36, da qual mantén geneticamente as características do perfil de proteína e aminoácidos (MANDARINO *et al*., 1992) e do reduzido teor de isoflavonas (CARRÃO-PANIZZI *et al*., 1998) e de sabor mais suave (CARRÃO-PANIZZI *et al*., 1999).

A soja BRS 258 apresenta melhor potencial e adaptação nas regiões acima de 600 m de altitude. È recomendada para semeaduras nos estados de PR, SP e SC (PIPOLO *et al*., 2005). Nas regiões abaixo de 600 m de altitude, deve ser semeada a partir de 25 de outubro. Apresenta alto teor de proteína de

aproximadamente de 41,70% e 23,70% de teor de óleo. A cor da flor é branca, o hilo marrom claro e os grãos graúdos, sendo adequada para o cultivo orgânico e para a alimentação humana, devido a seu sabor mais suave (EMBRAPA, 2008). Ambas cultivares foram obtidas através de colheita automotriz (Figura 2).

Figura 2. Beneficiamento do grão de soja.

3.Compostos bioativos no grão de soja com benefícios à saúde

Os peptídeos da semente de soja têm atividade antioxidante (PEÑA-RAMOS, 2002) e antiobesidade (NAKAMORY, 2002). As sementes de soja contêm também proteínas bioativas que possuem atividade anticâncer, incluindo lectinas (ABE, *et al.*, 1996) e o peptídeo conhecido como a lunasina (DE LUMEN & GALVEZ, 2002).

Os compostos bioativos podem se dividir em protéicos como a lunasina, o inibidor de Bowman Birkman (BBI) e a lectina, e os não protéicos como as isoflavonas e as saponinas.

3.1 Lunasina

A lunasina é um peptídeo único, isolado originalmente da soja e depois na cevada o qual pode prevenir alguns tipos de câncer (JEONG, 2003). Lunasina (Figura 3) é um peptídeo da soja com 43 resíduos de aminoácidos e contêm nove resíduos de ácidos aspárticos na sua extremidade (DE LUMEN, 2005). Foi descoberto de forma acidental por Alfredo Galvez, em 1996, como resultado da sua investigação sobre o perfil nutricional na proteína de soja no laboratório do Dr.

De Lúmen, na UC Berkeley. Esse peptídeo é encontrado em pequenas quantidades em sementes de soja e nos alimentos à base de soja, bloqueia a divisão celular pela ligação a proteínas específicas cromossomais chamadas "histonas desacetiladas" (SOY LABS, 2007).

SKWQHQQDSCRKQLQGVNLTPC**EKHIMEKIQGRGDDDDDDDDD**

Onde:

C = Cistina	I = Isoleucina	Q = Glutamina
D = Ácido Aspártico	K = Lisina	R = Arginina
E = Ácido Glutâmico	L = Leucina	S =Serina
G = Glicina	M = Metionina	T = Treonina
H = Histidina	N = Asparagina	V = Valina
	P = Prolina	W =Triptofano

Figura 3. Seqüência dos 43 aminoácidos da lunasina

Fonte: Lam *et al.*, 2003.

Sua eficácia contra compostos químicos oncogénos tem sido demonstrada em cultivos celulares e em um modelo do câncer de pele em ratos. Galvez *et al.*, (1997), isolaram e clonaram um cDNA que codifica uma albumina 2S processada pós-translacionalmente (Gm2S-1) da maturação média das sementes de soja. Este único peptídeo da soja com 43 aminoácidos, no qual a extremidade carboxílica contém nove resíduos de asparagina, e um terminal arginina-glicina-asparagina, modifica a adesão celular, e uma hélice com estrutura homóloga à região conservada das proteínas ligantes de cromatinas (GALVEZ *et al.*, 2001), são conhecidos agora como lunasina (GALVEZ & DE LUMEN, 1999). A lunasina da soja parece ter potencial como um novo agente anticancerígeno onde os agentes carcinogénicos são substâncias químicas (JEONG *et al.*, 2003). Pesquisas posteriores serão essenciais para confirmar estas observações preliminares e os possíveis beneficios à saúde, incluindo seu papel na prevenção de doenças crônicas.

Em 2004, foi publicado um estudo sobre a concentração de lunasina em diferentes genótipos de soja dos Estados Unidos. Nesta pesquisa descreveram o desenvolvimento de uma metodologia para a quantificação de lunasina pelo método imunoenzimático (ELISA), método para identificar e quantificar as variações na concentração de lunasina em 144 genótipos de soja selecionados da coleção de germoplasma de soja do Departamento de Agricultura dos Estados Unidos-USDA (GONZÁLES DE MEJÍA *et al*., 2004). Os resultados indicaram que com lunasina sintética e com anticorpo monoclonal, o método ELISA mostra uma boa reprodutibilidade com uma concentração linear dentro do intervalo de 24-72 ng/mL, um limite de detecção de 8 ng/mL, e uma valorização de 90% sobre as amostras de soja. Concentrações de lunasina nas amostras de soja testadas variam de 0,10 a 1,33 g/100 g de farinha. Diferenças, que ultrapassaram 100%, foram observadas entre os níveis de maturidade semelhantes que foram cultivadas em um mesmo ambiente, indicando que existem diferenças genéticas da soja para lunasina. Concentrado, isolado e hidrolisado protéico de soja contêm 2,81 ± 0,30, 3,75 ± 0,43 e 4,43 ± 0,59 g lunasina/100 g de farinha, respectivamente, enquanto a farinha de soja e soja em flocos contém 1,24 ± 0,22 g lunasina/100 g de farinha. Produtos enriquecidos com isoflavonas contêm muito pouca ou nenhuma lunasina. A massa relativa das amostras de lunasina foi de 5,45 ± 0,25 kDa. A ampla gama de concentrações de lunasina dentro da espécie *Glycine max*, indicam que os níveis deste importante polipeptídeo bioativo podem ser manipulado geneticamente. Além disso, as proteínas isoladas de soja e hidrolisados de soja contêm as maiores concentrações de lunasina (GONZÁLES DE MEJÍA *et al*., 2004).

3.2 Inibidor de Bowman-Birk (BBI)

O inibidor de Bowman-Birk (BBI) tem 71 resíduos de aminoácidos, peso molecular de 7,975 kDa, e sete pontes dissulfeto na molécula. Este inibidor inibe estequiometricamente 1 mole de tripsina e 1 mole de quimiotripsina de maneira independente e simultânea (BIRK, 1985). A seqüência de aminoácidos, mostrando

as pontes dissulfeto e os centros de complexação com as enzimas podem ser vistas na Figura 4.

O ponto isoelétrico do BBI é de pH 4,0 a pH 4,2. Os valores do ponto isoelétrico para diversos legumes e frutos variam entre pH 4,0 e pH 9,77. A variação é devida à diferença de resíduos de aminoácidos em diferentes BBI. O inibidor de Bowman-Birk é rico em resíduos do aminoácido cistina (cerca de 20%) (WEDER & HAUSSNER, 1991).

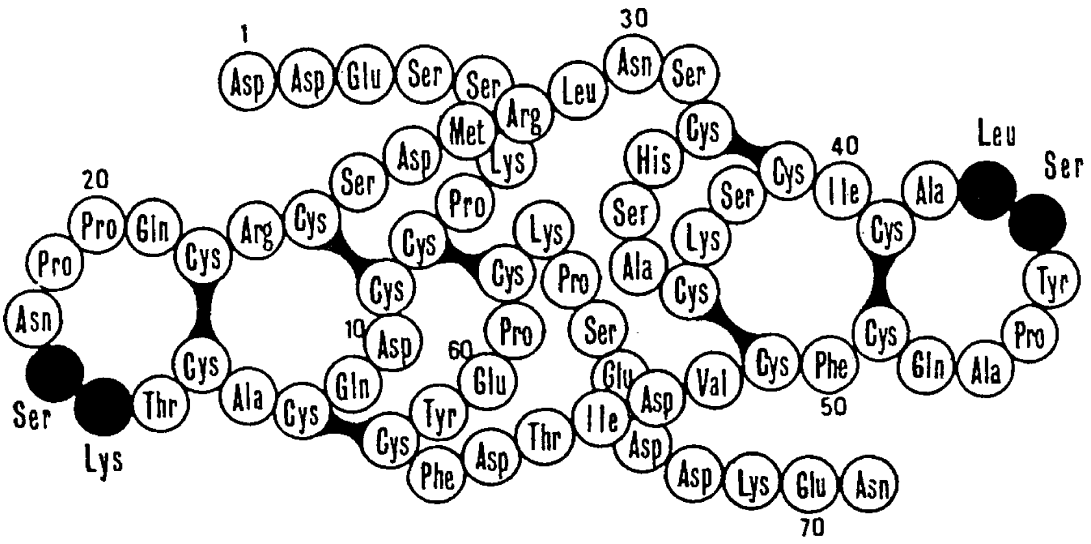


Figura 4. Sequência de aminoácidos do BBI, pontes dissulfeto e centros de ligação à tripsina (Ser, Lys) e à quimotripsina (Ser, Leu).

Fonte: Losso, 2008.

O BBI é estável dentro da faixa de pH encontrados na maioria dos alimentos, pode resistir à temperatura da água fervente durante 10 min, resistente a pH ácidos e da enzimas proteolíticas do trato gastrointestinal, é biodisponível e, não alergênico. Inibidores da protease, em geral, não são considerados ingredientes bioativos para a fortificação dos alimentos devido a sua incapacidade de ser muito específico. A FDA considera BBI como uma droga. A maioria dos compostos

bioativos previne doenças atuando na inibição da enzima que catalisa o processo patológico. Por isso BBI se enquadra na definição de um alimento funcional.

Em particular, o papel dos inibidores de protease de origem alimentar, como o BBI, está sendo reconhecido por pesquisadores biomédicos, colocando-os como potenciais agentes quimiopreventivos (WAN *et al.*, 2002; LIPPMAN & MATRISIAN, 2000; KENNEDY, 1998; MEYSKENS, 2001), especificamente nos casos de câncer de mama.

3.3 Lectina

As lectinas ou hemaglutininas podem ser caracterizadas e detectadas por sua habilidade em aglutinar eritrócitos, em certos casos com alta especificidade (LIS & SHARON, 1973; ASKAR, 1986). Todos estes efeitos são produzidos pela habilidade das lectinas de se ligarem a tipos específicos de açúcares na superfície celular (DESHPANDE & DAMODARAN, 1990). Além dessas propriedades, as lectinas podem promover estimulação mitogênica de linfócitos e aglutinação de células cancerosas (LIS & SHARON, 1973; LIENER, 1981).

A Hemaglutinina ou lectina na dieta de ratos mostrou uma redução significativa no ganho de peso em comparação aos controles, e também reduziu a digestibilidade do nitrogênio e retenção de nitrogênio na dieta, aumentando a perda de nitrogênio nas fezes e na urina (LI *et al.*, 2003). Foram detectados anticorpos para SBA no soro de aves, o que implica que o SBA permaneceu ativo no trato gastrointestinal (FASINA *et al.*, 2004).

As lectinas são acumuladas nas vacúolas das proteínas das sementes de armazenamento e nos cotilédones e são degradados durante a germinação das sementes (PUSZTAI, 1991; ORF *et al.*, 1979). A lectina apresenta várias propriedades anti-nutricionais, como também propriedades anticancerígenas (VASCONEZ-COSTA, 2004; GONZÁLES DE MEJÍA & PRISECARU, 2005). Em

estudos de casos em humanos, são utilizadas como agentes terapêuticos, ligando-se preferencialmente às membranas celulares de câncer ou seus receptores, causando citotoxicidade provocando câncer na célula por aglutinação e/ou agregação como pode ser observada na Figura 5 (GONZÁLEZ DE MEJÍA & PRISECARU, 2005). Algumas lectinas dietéticas podem causar um efeito quimiopreventivo no câncer de mama em humanos, inibindo o crescimento e proliferação celular in vitro (VALENTINER *et al.*, 2003).

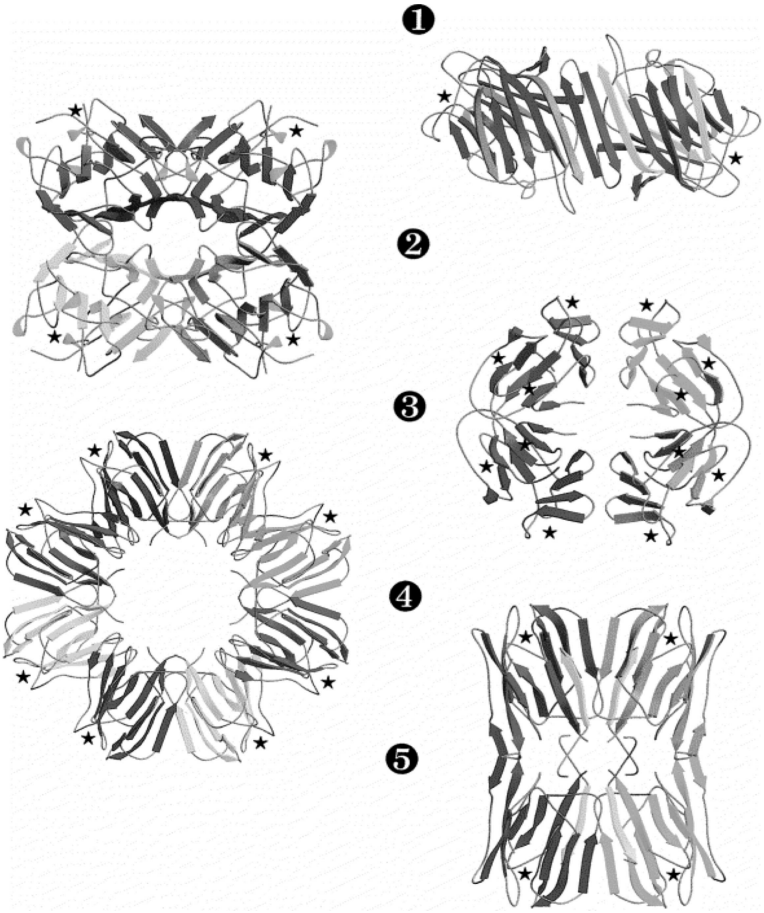


Figura 5. Organização oligomérica de diferentes lectinas que se ligam a manose. Estrelas (★) monossacarídeo obrigatório, (1) *Lathyrus ochrus* dímero, (2) Con A tetrâmero, (3) *Galanthus nivalis* tetrâmero, (4) Heltuba (*Helianthus tuberosus*) octâmero (5) Jacalin tetrâmero.

Fonte: Gonzáles de Mejía & Prisecaru, 2005.

3.4 Isoflavonas

Soja e seus derivados apresentam grande potencial no mercado de alimentos funcionais devido à presença de compostos bioativos como as isoflavonas, as quais têm sido largamente estudadas quanto a seus efeitos biológicos benéficos à saúde humana, tais como, atividade estrogênica (MURPHY, 1982), antiestrogênica (especialmente sobre os sintomas da síndrome do climatério e da osteoporose) (POTTER *et al*., 1998), antifúngica (NAIM *et al*., 1974) hipercolesterêmica (ANTHONY, 1996) e anticarcinogênica (SHAO *et al*., 1998), o que foi comprovado em populações asiáticas, em virtude de seu alto consumo de soja (MESSINA, 1997). Estas propriedades biológicas são predominantes quando as isoflavonas estão presentes na forma aglicona (sem glicose) em vez de β-glicosídeos (conjugadas à glicose) (LIGGINS *et al*., 2000, RIBEIRO *et al*., 2006).

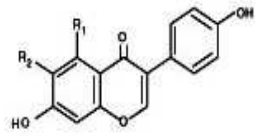
As isoflavonas são uma subclasse dos flavonóides, que por sua vez pertencem ao grupo dos chamados fitoquímicos, compostos não incluídos como nutrientes, porém que vêm chamando a atenção de pesquisadores devido às suas atividades estrogênicas e propriedades de prevenção contra câncer e outras doenças crônicas.

Os flavonóides incluem todos os compostos fenólicos de uma planta e sua estrutura básica consiste em 2 anéis de benzeno ligados por um anel pirano heterocíclico. Outros exemplos de flavonóides são: antocianinas, flavonas, flavonóis, flavanóis, auronas e calconas. As isoflavonas compreendem 12 isômeros, mostrados na Figura 6. A soja é a única fonte da natureza que contém grande quantidade de isoflavonas, acima de 3 mg/g em base seca. As isoflavonas originais presentes no grão de soja são a genisteína, a daidzeína e seus respectivos β-glicosídeos conjugados. Em menores quantidades, também se encontram a gliciteína e glicitina (LIU, 1997).

A concentração de isoflavonas nos grãos de soja é geneticamente controlada e influenciada pelas condições ambientais, sendo a temperatura durante o desenvolvimento do grão o fator mais importante (CARRÃO-PANIZZI *et*

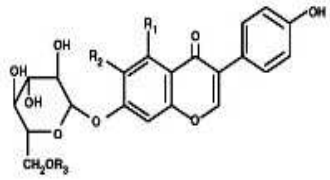
al., 1988; TSUKAMOTO *et al*., 1995). A presencia e a concentração das isoflavonas nos produtos à base de soja dependem das condições de processamento, principalmente a temperatura de tratamento do material (COWARD *et al*., 1988; WANG & MURPHY, 1996).

Em produtos de soja não-fermentados, estão presentes as isoflavonas na sua forma conjugada, enquanto que em produtos fermentados predominam as agliconas. Isso devido à presença da enzima β-glicosidase, produzida pelos microrganismos responsáveis pela fermentação, que hidrolisa a ligação glicosídica da molécula, resultando em aglicona. Produtos não-fermentados têm concentrações de isoflavonas duas a três vezes maiores que produtos fermentados (WANG & MURPHY, 1996).



aglicon

R_1	R_2	Isômeros
H	H	Daidzeína
OH	H	Genisteína
H	OCH_3	Gliciteína



β-glicosídeo

R_1	R_2	R_3	Isômeros
H	H	H	Daidzina
OH	H	H	Genistina
H	OCH_3	H	Glicitina
H	H	$COCH_3$	6"-*O*-Acetildaidzina
OH	H	$COCH_3$	6"-*O*-Acetilgenistina
H	OCH_3	$COCH_3$	6"-*O*-Acetilglicitina
H	H	$COCH_3COOH$	6"-*O*-Malonildaidzina
OH	H	$COCH_3COOH$	6"-*O*-Malonilgenistina
H	OCH_3	$COCH_3COOH$	6"-*O*-Malonilglicitina

Figura 6. Estrutura química dos 12 isômeros de isoflavonas presentes na soja.

Fonte: Liu, 1997.

Na tabela 5, Mandarino *et al*., (2006), apresentam o teor de isoflavonas em diversas cultivares de soja desenvolvidas pela Embrapa Soja, sendo que a cultivar BRS 133 apresenta o maior teor de isoflavonas. A cultivar BRS 258, apresenta o menor teor de isoflavonas, como a cultivar BR-36, que lhe deu origem, confirmando a característica genética dessa cultivar (CARRÃO-PANIZZI *et al*., 1998).

Tabela 5. Teores totais de isoflavonas (mg/100g) em amostras de sementes de diferentes cultivares de soja, semeadas em outubro, novembro e dezembro, safra 2004/2005.

	Época de Semeadura		
Cultivares	Outubro	Novembro	Dezembro
BRS 133	219,09 a A	160,81 a B	171,55 d C
BRS 185	215,92 b A	168,84 c B	145,91 c C
BRS 260	206,08 c A	181,69 b B	108,52 f C
BRS 214	195,27 d A	152,20 e C	159,36 bB
BRS 261	193,22 d A	118,02 gh B	79, 37 h C
BRS 213	191,63 d A	145,18 ef B	104,90 f C
BRS 184	169,31 e A	144, 88 ef B	129,05 e D
BRS 259	150,03 f A	97,74 i C	109,00 f B
BRS 215	146,86 fg A	90,60 i C	123,36 e B
EMBRAPA 48	143,95 fg A	73,50 j C	91,89 g B
BRS 257	140,35 g A	123,24 g B	93,33 g C
BRS 232	120,87 h A	66,13 jk B	72,31 h B
BRS 231	120,61 h A	113,49 h B	90,06 g C
BRS 262	110,64 i C	139,65 f B	165,87 ab A
BRS 230	81,56 j A	62,34 k B	56,47 i B
BRS 258	56,37 k A	47,95 l B	37,86 j C

Médias repetidas pelas mesmas letras minúsculas nas linhas e mesmas letras maiúsculas nas colunas não diferem entre si pelo teste de tukey ($p < 0,05$).

Fonte: Mandarino *et al*., 2006.

3.5 Saponinas

As saponinas são triterpenóides naturalmente encontrados em muitos alimentos derivados de uma grande variedade de espécies vegetais (PRICE *et al*., 1987). Eles são metabólitos secundários de plantas contendo um esteróide ou triterpenóide aglicona com um número de moléculas de carboidrato ligados através de conexões éteres e ésteres em uma ou mais sítios de glicosilação. Sementes da soja (*Glycine max* L. Merrill) contêm entre 0,6% a 6,5% (b.s.) de saponinas triterpenóides dependendo da variedade, ano de cultivo, local, e grau de maturidade. As saponinas possuem atividade antifúngica, antiviral, espermicida, expectorante, diurética e antiinflamatória (BERHOW *et al*., 2006) e atividade hipocolesterolêmica (POTTER, 1995; LEE *et al*., 2005).

As saponinas da soja foram divididas em um grupo de saponinas da soja no grupo B e respectiva base de estrutura aglicona. A saponinas da soja (grupo B) parece que existam no tecido vegetal intacta como conjugados de 2,3-dihidro-2, 5 - dihidroxi-6-metil-4*H*-pirano-4-um (DDMP), na posição 22 hidroxila (KUDOU *et al*., 1993). O DDMP conjugados é relativamente lábil e são facilmente degradados, resultando na formação de não-DDMP grupo B das saponinas da soja. As várias outras formas do grupo B das saponinas da soja podem surgir a partir de açúcares suplentes nos oligossacarídeos anexados à posição de 3-hidroxil ou aglicona. As saponinas da soja do grupo A estão com alternadas posições didesmosidicas de açúcares em ambos grupos de oligossacarídeos anexado à aglicona nas hidroxilas de posições de 3 e 21 (SHIRAIWA, 1991). Várias outras formas saponinas, incluindo o Grupo E das saponinas da soja estão em um número menor de açúcar da cadeia saponinas, estes são provavelmente resultantes da extração e das etapas do processamento (HENG *et al*., 2006).

Berhow (2006) publicou um resumo das 20 diferentes formas de saponinas de soja e seus produtos transformados. As estruturas e a nomenclatura das saponinas da soja são mostradas na Figura 7 e Tabela 6, respectivamente.

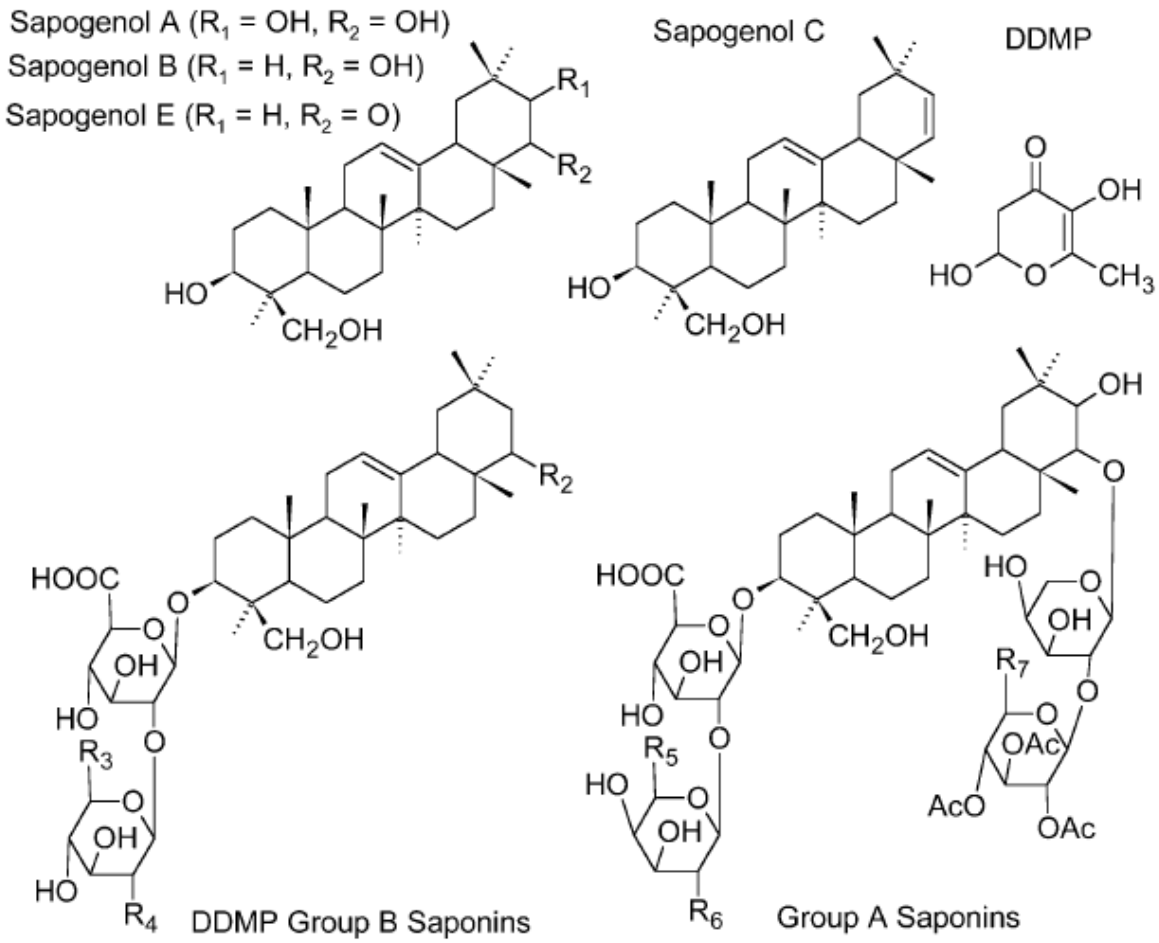


Figura 7. Estrutura das saponinas da soja.

Fonte: Berhow, 2006.

Tabela 6. Nomenclatura das saponinas da soja.

Grupo B		MW	R_2	R_3	R_4
Saponina I	(Bb)	942	OH	CH_2OH	*O*-β-D-Glucose
Saponina II	(Bc)	912	OH	H	*O*-α-L-Rhamnose
Saponina III	(Bb')	796	OH	CH_2OH	OH
Saponina IV	(Bc')	766	OH	H	OH
Saponina V	(Ba)	958	OH	CH_2OH	*O*-α-L-Rhamnose
Saponina	(Be)	940	O	CH_2OH	*O*-β-D-Glucose
Saponina	(Bd)	956	O	CH_2OH	*O*-α-L-Rhamnose
Saponina βg		1068	*0-DDMP*	CH_2OH	*O*-β-D-Glucose
Saponina βa		1038	*0-DDMP*	H	*O*-α-L-Rhamnose
Saponina γg		922	*0-DDMP*	CH_2OH	OH
Saponina γa		892	*0-DDMP*	H	OH
Saponina αg		1084	*0-DDMP*	CH_2OH	*O*-α-L-Rhamnose
Grupo A		**MW**	**R_2**	**R_3**	**R_4**
Saponina aA_1	(Ab)	1436	CH_2OH	*O*-β-D-Glucose	CH_2OAc
Saponina aA_2	(Af)	1274	CH_2OH	OH	CH_2OAc
Saponina aA_3		1202	H	OH	CH_2OAc
Saponina aA_4	(Aa)	1364	CH_2OH	*O*-β-D-Glucose	H
Saponina aA_5	(Ac)	1202	CH_2OH	OH	H
Saponina aA_6		1172	H	OH	H
Saponina aA_7	(Ac)	1420	CH_2OH	*O*-β-L-Glucose	CH_2OAc
Saponina aA_8	(Ad)	1406	H	*O*-β-D-Glucose	CH_2OAc

Fonte: Berhow, 2006.

4. Germinação

4.1 Definição

A germinação de sementes é definida como o processo pelo qual, sob condições favoráveis, o eixo embrionário retorna ao seu desenvolvimento, que tinha sido interrompido por ocasião da maturidade fisiológica. A absorção de água na semente é o primeiro evento da germinação e promove a reidratação dos tecidos, o aumento da respiração e de outras atividades metabólicas, que culminam com o fornecimento de energia e de nutrientes necessários para a retomada do crescimento da parte do eixo embrionário (NETO, 2004).

Para que uma semente possa germinar, certas condições têm que haver condições favoráveis, tais como: fornecimento adequado de água, temperatura desejável, certa composição de gases na atmosfera, luz (certas sementes) e ausência de inibidores da germinação. As duas primeiras condições são os fatores mais cruciais (CARVALHO & NAKAGAWA, 1988).

4.2 Metabolismo e fases do processo germinativo

A germinação é um processo que envolve tanto reações catabólicas, como a degradação de substâncias de reserva, quanto reações anabólicas na produção de novas células e organelas do embrião (METIVIER, 1979).

CARVALHO & NAKAGAWA (1988), descreveram detalhadamente as três fases do processo germinativo das sementes, em função do teor de umidade:

Fase I: seria de forma geral muito rápida (em uma ou duas horas a semente a completaria), atingindo um teor de umidade oscilando entre 25-30% para as sementes de cereais e leguminosas. Fisiologicamente, esta fase caracteriza-se por um acentuado aumento na intensidade respiratória (resulta na produção de grandes quantidades de energia, a qual, em boa parte, vai ser utilizada em uma série de reações bioquímicas), principalmente a partir de 14-16%

de umidade (AHRENS & PESKE, 1994). Bioquimicamente, caracterizam-se pelo início da degradação das substâncias de reserva (carboidratos, proteínas e lipídios) que deverão nutrir o crescimento do eixo embrionário até o ponto em que a plântula resultante tenha desenvolvido um sistema radicular capaz de retirar do solo os nutrientes que a planta necessita. Além destas, os fosfatos, embora em quantidades relativamente pequenas, são de vital importância, pela sua participação na composição das moléculas armazenadoras de energia. O transporte dessas substâncias exige que elas estejam desdobradas em substâncias de menor tamanho molecular.

Fase II : teria início ao atingirem-se valores de umidade entre 25-30%, em que estaria ocorrendo um transporte ativo das substâncias desdobradas na fase anterior, do tecido de reserva para o meristemático. Nesta fase, embora recebendo algum nutriente, o eixo embrionário ainda não consegue crescer e a semente praticamente pára de absorver água. A duração desta fase é de 8 a 10 vezes mais longa que a primeira, e a intensidade respiratória da semente também aumenta de maneira muito lenta.

Fase III : subitamente, a partir de um teor de umidade que varia de 35 a 40%, a semente volta a absorver água e respira intensamente. Deste ponto em diante tem início o crescimento visível do eixo embrionário, e inicia-se a fase 3 da germinação. Ao nível bioquímico, o que a caracteriza é que as substâncias desdobradas na fase 1 e transportadas na fase 2 são organizadas em substâncias complexas, para formar o citoplasma, o protoplasma e as paredes celulares, o que permite o crescimento do eixo embrionário (brotamento). O início de uma nova fase não inibe a ocorrência da anterior, de modo que, quando a fase 3 se inicia, a semente em germinação apresenta simultaneamente as três fases.

Estudos de três cultivares de soja germinada por três dias demonstram que os teores de proteína alcançaram valores máximos após 48 horas do início da

germinação, sendo observado que a germinação além de ter induzido ao aumento do conteúdo protéico, causou redução do nível de atividade específica da lipoxigenase (BORDINGNON *et al.*, 1995).

A germinação de sementes proporciona aumento de seu valor nutritivo, pela melhoria da digestibilidade protéica e pelo aumento do valor do quociente de eficiência protéica (QEP), redução dos fatores antinutricionais nas leguminosas, tais como inibidores proteolíticos e lectinas, hidrólise de oligossacarídeos (rafinose e estaquiose) presentes na soja, os quais são causadores de flatulência. A germinação também proporcionou aumento da metionina, aminoácido limitante da proteína de soja (BARCELOS *et al.*, 2002).

Durante o processo de germinação, as enzimas existentes na semente, entre elas as fitases, são rapidamente ativadas por simples hidratação. Com a ativação das fitases, o ácido fítico é hidrolisado, liberando H_3PO_4, Mg^{2+}, Ca^{2+}, e inositol. Conseqüentemente, durante a germinação ocorrem reduções nos teores de acido fítico, o qual possivelmente aumentará a biodisponibilidade dos minerais.

4.3 Germinação dos grãos de soja

A soja apresenta elevado valor nutritivo, que é determinado por sua composição protéica. Entretanto, a semente apresenta em sua estrutura fatores antinutricionais que podem interferir na disponibilidade de nutrientes, resultando em inibição de crescimento, hipoglicemia ou danos a tecidos, como pâncreas ou fígado. Entre estes constituíntes, destacam-se o ácido fítico e os inibidores de tripsina (LIENER, 1981).

Para melhorar a qualidade nutricional da soja e utilizá-la como alimento, há necessidade de remover ou inativar esses constituintes indesejáveis. A criação de cultivares através de manipulação genética, que contêm pequenas ou nenhuma quantidade desses constituintes indesejáveis é uma alternativa, porém requer estudos prolongados sobre a natureza química e bioquímica destes compostos, bem como as conseqüências agronômicas de rendimento da colheita, tolerância

ao solo, necessidade de luz e água e resistência a pragas (SATHE & SALUNKHE, 1984). Outras formas de redução de componentes indesejáveis seriam os processos como moagem, hidratação, cozimento, fermentação, extração com solvente e germinação (RACKIS *et al*., 1979; BRESSANI, 1983; ABDULLAH *et al*., 1984; MOSTAFA & RAHMA, 1987; BELÉIA *et al*., 1990).

O processo de germinação tem sido proposto como uma alternativa para melhorar as qualidades nutricionais da soja (MOSTAFA & RAHMA, 1987). Neste processo são reportadas reduções nos teores de ácido fítico dependendo do tempo de germinação e da cultivar estudada (ABDULLAH *et al*., 1984; SUPARMO & MARKAKIS, 1987).

Os efeitos da germinação em sementes de soja sobre a composição química, constituíntes bioquímicos e fatores antinutricionais podem variar grandemente com as condições de germinação (temperatura, luz, umidade e tempo), variedades ou cultivares das sementes e os métodos analíticos (BAU *et al*., 1997). O desenvolvimento de produtos alimenticios provenientes da germinação da soja pode ser outra forma de aumentar ainda mais a versatilidade e utilidade de este grão.

Na tabela 7, são apresentadas algumas condições de germinação da soja usadas em laboratorio segundo diferentes autores.

Tabela 7. Germinação da soja: pré-tratamentos, maceração, germinação e secagem, segundo diferentes autores.

Pré-Tratamento	Maceração (duração)	Temperatura de germinação	Tempo de germinação	Umidade relativa	Secagem (T℃/duração)	Observações	Referências
-	3 h/ 50℃	25℃	72 h	-	-	-	Bau & Debri, 1979
-	72 hs/ 41℃	30℃ / 20℃	8h a 30℃	100%	-	Germinação entre papéis	Egli *et al.*, 2005
-		24 ± 0,5 ℃	24,48,72,96 h	92 ± 2%	-	Germinação entre papeis.	Gloria *et al.*,2005
-	27℃ ± 2 ℃	25℃ ± 2	0,48,96 h	-	60 ℃ / 24 h	Em algodão	Jyothi *et al.*, 2007
-	-	20℃	0,6,12,18,24,30, 36,48,72,96 h	-	60 ℃ / 24h	-	Kim *et al.*, 2005
-	-	25 ℃ e 30 ℃	0,24,48,72,96, 120, 144h	100 %	Liofilização	-	Kumar *et al.*, 2006
NaOCl 0,7%	6 h a T°amb	25℃	96 h	-	Liofilização	-	Martin-Cabrejas *et al.*, 2008
-	-	25℃	0,6,12,18,24,30, 42,48,54,60h	100%	Liofilização e armazenamento a –20℃.	Germinação entre papéis	Ribeiro *et al.*, 1999
-	-	25℃ ± 1	0,6,12,18,24,30, 36, 42,48,54,60, 64,68,72h	100%	Liofilização	-	Ribeiro *et al.*, 2006
-	-	T°amb.	12,24,48,72 h	100%	Liofilização	-	Suberbie *et al.*, 1981
Lavagem 3 vezes	T°amb / 12 h	40℃	Até que o cumprimento do hipocotilo seja de 0,5; 2,5 e 6,5 mm	100%	Liofilização	-	Zhu *et al.*, 2005

4.4 Efeitos do processo de germinação dos grãos de soja no seu conteúdo de compostos bioativos

São poucas as pesquisas que relatam os efeitos do processo de germinação da soja no seu conteúdo de compostos bioativos. Atualmente, no mundo não existem pesquisas sobre o efeito da germinação do grão de soja no conteúdo da lunasina. Desta forma este é o primeiro estudo a avaliar as mudanças no conteúdo de lunasina pelo processo de germinação.

Com relação aos inibidores de tripsina como o BBI, durante a germinação, os resultados encontrados são contraditórios, com relatos de aumento (JIMENEZ *et al*., 1985), redução (BATES *et al*., 1977; BAU & DEBRY, 1979; MOSTAFA & RAHMA, 1987), ou pouca alteração em sua atividade (COLLINS & SANDERS, 1976).

No caso da lectina, Chen *et al*., 1977, mostrou um rápido desaparecimento da atividade de hemoaglutinação em extratos de soja após 4 dias de germinação e Nielsen & Liener, 1988, relatam uma diminuição na atividade de hemoaglutinação durante a germinação do feijão.

Zhu *et al*., (2005) realizaram uma pesquisa para analisar o conteúdo de isoflavonas em sementes de soja germinada variedade Hutcheson Caviness e encontraram que, o conteúdo total da isoflavonas aumentou rapidamente durante a fase inicial de germinação. Os valores máximos de isoflavonas totais foram observados quando os comprimentos da radícula foram entre 0,5 e 2,5 mm. Uma diminuição no conteúdo de isoflavona foi observada após esta etapa. O aumento foi dominado pelos β-glicosídeos conjugados, especialmente nas formas malonil glicosídeos. O conteúdo de agliconas, genisteína e daidzeína, atingiu concentrações mais elevadas, logo após a imersão. Um processo de germinação controlado pode ser utilizado para melhorar o conteúdo de isoflavonas em soja.

Porém, o máximo de benefícios da soja como nutracêuticos pode ser alcançado quando o cumprimento do hipocótilo é de cerca 0,5 a 2,5 milímetros.

Ribeiro *et al*., (2006), estudaram a atividade da β-glucosidase e o conteúdo de isoflavonas nas radículas e nos cotilédones de grãos de soja germinados da cultivar BRS 213 por 72 horas a 25 ℃, com amostras coletadas e analisadas a cada 6 horas e referem que, a germinação de soja afeta a atividade das β-glucosidases, o total de isoflavonas e o conteúdo de suas formas isoméricas. Também, demonstraram que, durante a germinação a atividade β-glucosidase é aumentada na radícula e no cotilédon, enquanto que o teor de isoflavonas totais aumentou nos cotilédones e teve uma diminuição nas radículas. Assim, as alterações no conteúdo de isoflavonas dependerão da fase de germinação das sementes de soja e de seu metabolismo fisiológicos.

Shimoyamada & Okubo (1991), descreveram que as saponinas da soja podem atingir um nível de 0,5% e que durante a germinação se produz um grande aumento no conteúdo destas saponinas, encontrando-se que após oito dias de germinação, a concentração de saponinas é oito vezes maior do que nas sementes sem germinar.

Além disso, Jyothi *et al*., (2007), relata que durante a germinação da soja por 4 dias, o conteúdo de saponinas aumentou de 2,8% para 8,9% em grãos de soja maduros.

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Capítulo 2

Bioactive compounds and chemical composition of two Brazilian soybean cultivars with low (BRS 133) and high (BRS 258) protein contents

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This paper was submitted to Journal of Agricultural and Food Chemistry on Fev 5th, 2009

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Abstract

Soybean is a major source of protein and other nutrients with health benefits to a great part of the world population. Brazil produced in the last growing season 2007/2008 61.5 million tons of soybeans, or approximately 27% of the world production, while US production was approximately 84 million tons. Soybeans are characterized by having low content of or none starch, about 20% oil and nearly 40% protein, both considered to have high quality. Soybean is a complex matrix containing several bioactive compounds, including lunasin, Kunitz (KSTI) and Bowman-Birk Inhibitors (BBI), isoflavones, saponins, and bioactive peptides. The objective of this study was to determine the composition of nutrients and bioactive compounds of two Brazilian soybean cultivars with low and high protein contents, BRS 133 and BRS 258, respectively. The two cultivars studied exhibited a typical soybean chemical composition. The high protein cultivar, however, exhibited 17% lower carbohydrate content and lower chemical score (63.0) in relation to the low protein cultivar with higher chemical score (76.0), an advantage associated with the higher content of methionine (1.22%) of the low protein cultivar BRS 133, compared to the 1.01% found in cultivar BRS 258. In contrast, cultivar BRS 258 had 15.48, 30.05, 18.65, 9.03 and 11.45% more calcium, phosphorus, iron, copper and zinc, correspondingly, than BRS 133. BRS 258 also exhibited higher concentrations of lunasin, BBI and lectin (20.26, 19.01 and 27.14%), respectively, than cultivar BRS 133. In addition, the BRS 133 had 75.38% higher amounts of total isoflavones (5.08% of total aglycones) and 31.04% total saponins, as compared to BRS 258.

Keywords: soybean, bioactive compounds, lunasin, BBI, lectins, saponins, isoflavones, chemical score.

1. Introduction

Soybean (*Glycine max* L. Merril) is a legume consumed worldwide. Soybean foods have generated a lot of interest because of its beneficial effects on nutrition and health. Studies have shown that Asian populations habitually consuming soybean products have a lower risk of osteoporosis and some chronic diseases, most notably heart disease and cancer (*1*).

Bioative compounds in vegetables vary greatly with the plant species, cultivars, weather and geographical location. Soybean is a complex food matrix containing little or no starch, about 20% oil and 40% high quality protein (*2*), in addition to several important bioactive compounds, including lunasin, Bowman Birk Inhibitor (BBI), isoflavones, saponins, and other soy proteins and bioactive peptides. Lunasin is a novel 43 amino acid polypeptide cancer preventive peptide originally isolated from soy (*3, 4*). BBI is a 71 amino acid protein with 7 disulfide bonds, which stabilizes an active configuration, and has a double head with the chymotrypsin inhibitor domain located on one of the heads (*5*). Lectins in turn are known for having both anti-nutritional and anti-carcinogenic properties (*6, 7*). The lectins accumulate in protein storage vacuoles of the cotyledons and are degraded during seed germination and maturation (*8, 9*). Lipids are an important source of the compounds responsible for flavor in soybean protein products. Soybean seeds are a major source of genistein, daidzein and glycitein and the corresponding glycosides genistin, daidzin and glycitin, and their malonyl and acetyl conjugates. The isoflavone glycosides are present primarily as β-glucosides and a portion of the glucosides are substituted on the C-6 hydroxyl of the glucose by a malonyl group, especially in the seed hypocotyls (*10*). Saponins are plant glycosides whose aglycone structure is either a triterpenoid or a steroiod molecule.

Based on the available scientific evidence, the US FDA (Food and Drug Administration) allowed American manufacturers of soybean products to make health claims for as long as a minimum of 6.25 g of soy protein is present in a regular portion of the food product, in addition to having low saturated fat and

cholesterol contents (*11*). The minimum protein content is based on the consideration that the beneficial effect is attributed to the protein fraction and that a minimum daily intake of 25 g of soy protein should be necessary for the health effect to be significant (*12,13*).

The objective of this study was to determine the nutritional composition of two Brazilian soybean cultivars BRS 133 and BRS 258, and their proteinaceous bioactive compounds: lunasin, Bowman-Birk inhibitor (BBI) and lectin, and the non-proteinaceous bioactive compounds, isoflavones and saponins.

2. Material and Methods

2.1 Material

The breeding program of Embrapa Soybean, Brazil has developed the conventional low-protein cultivar BRS 133 and the high-protein cultivar BRS 258 (*14*). BRS 133 was produced in the region of Ponta Grossa, while cultivar BRS 258 was produced in the region of Guarapuava. Both regions are in Paraná State, Brazil at 2007 and were provided by Embrapa Transferência de Tecnologia, Brazil.

The soybeans were sanitized for 10 min. with sodium hypochlorite solution (100 mg/Kg) and immediately washed three times with distilled water. The sanitized grains were frozen at – 30 ℃ for 4 hours , freeze-dried and milled. Whole soybean flours were obtained in a refrigerated hammer mill, model 680 from Marconi (Piracicaba, Brazil), and the powders stored at 7℃, conditioned in air-tight glass.

Immunoaffinity purified lunasin (98%) from soy and rabbit polyclonal antibody against the lunasin epitope –EKHIMEKIQGRGDDDDD were provided by Dr. Ben O. de Lumen, of the University of California at Berkeley. Purified A and B group soy saponins were prepared in the Peoria laboratory (USDA) (*15*). The primary polyclonal antibody specific for lectin from soybean was provided by Dr. Theodore Hymowitz from the Department of Crop Sciences, University of Illinois at Urbana-Champaign, USA. The lectin anti-serum was obtained at his laboratory by

immunizing young male New Zealand white rabbits with 5 mL subcutaneous injections of an emulsion containing 5 mg of pure lectin, 1 mL of distillated water and 1 mL of Freund's complete adjuvant. Six weeks after the first immunization, rabbits showing response to the antibodies (measured 20 days after the first injection) were injected again with a similar dose and bled two weeks later (*9*).

2.2 Determination of the weight of 1000 soybeans seed

The weight of 1000 seeds was determined by weighting eight replicates of 100 seeds each (*16*).

2.3 Proximal composition

Moisture, total proteins and ash of the whole flour (WSF) were determined by the AACC procedures 44-15, 46-13 and 08-12, respectively (*17*). The conversion factor 5.71 for protein was used. Lipids, total sugars and starch were determined according to the methods of the Adolfo Lutz Institute (*18*). Total carbohydrates, including total fiber, were determinated by inferred difference. Dietary, soluble and insoluble fibers were determined following the AOAC procedure 991.43 (*19*). Metabolizable energy of the flours was estimated by multiplying the protein and carbohydrate contents by 4 kcal per gram and fat by the factor of 9 kcal per gram.

2.4 Physical and physicochemical characteristics

Color was determined by means of a Color Quest II Hunterlab instrument (Reston, VA), determining the components L* (lightness), a* (green - / red +) and b* (blue - / yellow +), according to the CIE-L*a*b* system. The chrome (C*) and hue angle (h*) values were calculated as described by Minolta (*20*). The chrome value was calculated as shown in Equation 1, and the saturation angle as shown in Equation 2.

$$Chrome\ (C^*) = [(a^*)^2 + (b^*)^2]^{1/2} \qquad \text{(Equation 1)}$$

$$h = tan^{-1} (b^*/a^*) \qquad \text{(Equation 2)}$$

Particle size was determined in a Granutest, Model 295 instrument, according to procedure 965.22 of the AOAC (*19*).

For pH, procedure 943.02 of the AOAC (*19*) was followed and the water activity was determined in triplicate using an AquaLab, series 3, Model TE equipment.

2.5 Fatty acid composition

The Hartman & Lago procedure (*21*) was followed for the esterification step, and gas chromatography (Agilent series 6850 CGC system) for the analysis of the fatty acid methyl esters, using a capillary column (Agilent DB-23; 50% cyanopropyl-methylpoly-siloxane; 60m x 0.25mm). Instrument operating conditions were: detector temperature (280ºC), injector temperature (250ºC), oven temperature (110ºC) for the first 5 min, followed by increases of 5 ºC/min to reach 215 ºC and holding the temperature at 215 ºC for 24 min. Helium was used as carrier gas, and the injection volume was 1.0 µL, split 1:50.

The iodine index was determined following procedure Cd 1d-92, and the saponification index, procedure Cd 1c-85, both of the AOCS (*22*) and calculated on the basis of the fatty acid composition.

2.6 Total and free amino acid composition

After a 24h hydrolysis in 6M HCl/phenol at 100 ℃, the amino acids were reacted with phenylisothiocyanate *(23)* and the derivatives chromatographed using a Luna C-18, 100 Å; 5 µ, 250 mm x 4.6 mm (00G-4252-EQ) column, at 50 ℃. Quantification was carried out by comparison with a standard mixture and DL-2-aminobutyric acid was used as an internal standard from Sigma-Aldrich Corp, St Louis, MO; *(24).* Running time was 24 minutes. The free amino acids were

determined by extracting 1.25 g flour samples in 80% ethanol solution with 0.1M HCl, with 500 µL of a-aminobutyric acid, added as internal standard, in a 5 mL volumetric flask. The mixture was sonicated for 10 minutes and further homogenized for 1 hour, followed by centrifugation at 8,500 g for 15 minutes. The supernatant was filtered through a 0.22 mm membrane and a 40 µL aliquot derivatized as described above, for the injection of 20 µL into the above mentioned liquid chromatograph.

2.7 Minerals

Duplicate samples were calcinated at 500ºC to determine the dry ashing. Calcium and microelements were determined by atomic absorption spectrometry, according to procedure 968.08 of the AOAC (*19*) in a Metrolab equipment, Model 250. Phosphorus was determinated by spectrophotometry in UV Hitachi U-2000 equipment (*25*).

2.8 Protein extraction

The protein extraction procedure consisted in placing 50 mg of soybean flour and 1 mL of the extracting buffer (0.05M Tris-HCl pH 8.2) in an Eppendorf tube. After mixing, the samples were placed in an ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT) for 70 min, mixing them at every 10 min to avoid settlement. The water temperature was adjusted to 40 ℃ using a recirculation bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, NH). Following extraction, the samples were centrifuged at 20,000 g for 40 min at 8 ℃ in an Eppendorf centrifuge (Brinkmann Instruments, model 5417R, Westbury, NY), and the obtained supernatant was transferred to a new Eppendorf tube.

2.9 Determination of soluble protein concentration by DC assay

The protein concentration was determined using the Bio-Rad DC Microplate Assay Protocol (Bio-Rad Laboratories, Hercules, CA). Briefly, 5 µL of samples

(1:20 dilution) were placed in a 96-well plate and treated with 25 µL of reagent A and 200 µL of reagent B (Bio-Rad Laboratories, Hercules, CA). The plate was gently agitated and incubated for 15 min at room temperature. After incubation, the absorbance was measured at 630 nm. The protein concentration was calculated using pure bovine serum albumin standard curve ($y = 0.0002x - 0.0021$, $R^2 = 0.997$).

2.10 Enzyme-linked immunosorbent assay (ELISA) for lunasin and BBI

Lunasin concentration in soy flour was determined by ELISA (*3*) with the following modifications: 100 *µ*L of protein extracts (1:5,000 dilution) were placed in a 96-well plate and stored overnight (14 h). Lunasin mouse monoclonal antibody (1:4,000 dilution) was used as first antibody and anti-mouse IgG alkaline phosphatase conjugate (1:7,000) from Sigma-Aldrich Corp, St Louis, MO as the secondary antibody. The reaction was stopped adding 25 µL NaOH (3 N) at 30 min and the absorbance read at 405 nm after 35 min. Similar procedure was used for BBI analysis, samples of 100 µL of protein extracts (1/10,000 dilution) were placed in a 96-well plate, except that BBI mouse monoclonal antibody (1:1,000 dilution), Agdia, Inc., Elkhart, IN, was used as the first antibody and anti-mouse alkaline phosphatase (AP) conjugated IgG (1:2,000) Sigma-Aldrich Corp, St Louis, MO as the secondary antibody. Standard curves were determined using purified lunasin ($y = 0.0054x + 0.001$, $R^2 = 0.993$) and purified BBI ($y = 0.0108x + 0.0465$, $R^2 = 0.998$).

2.11 Western blot procedures

Identity of lunasin was established by Western blot analysis in protein extracts of soybean flour. Samples were centrifuged (20,000 g) at 8 ℃ to eliminate any precipitate. Unstained gels were soaked in 20 mL of blotting buffer (20% methanol, 80% Tris-glycine SDS) for 15 min. A Western blot sandwich was assembled by the following order: a sponge, filter, gel, polyvinylidene difluoride

(PVDF) membrane Inmobilon^{TM}-FL (Millipore Corporation), and another filter and sponge, being careful to avoid formation of bubbles, and then developed for 1 h at 110 V at 4 ℃. After the complete transfer, membran e was then saturated by incubation in 5% nonfat dry milk (NFDM) in 0.01% TBST (0.1% Tween 20 in Tris-Buffered saline) buffer for 1 h at 4 ℃, and washed three times for 5 min with fresh changes of 0.01% TBST. The washed gel was incubated with lunasin mouse monoclonal antibody (1/1000 dilution) prepared in 1% NFDM and TBST buffer for 16 h at 4 ℃. After washing the incubated membrane, the membrane Inmobilon TM-FL (Millipore Corporation) was incubated with anti-mouse IgG alkaline phosphatase conjugate (1/10,000 dilution) prepared in 1% NFDM in TBST buffer for 3 h at room temperature. The membrane was prepared for detection using chemiluminescent reagent, 500 µL of solution A and 500 µL of solution B (Lumigen TM, GE Healthcare, Buckinghamshire, UK).

2.12 Enzyme-linked immunosorbent assay (ELISA) for lectin

Lectin concentration in soy flour was determined by ELISA (*6*) with the following modifications. One hundred microliters (100 µL) of protein extracts (1:10,000 dilution) were placed in a 96-well plate. Lectin mouse polyclonal antibody (1:500 dilution) was used as the first antibody, and anti-rabbit IgG alkaline phosphatase conjugate (1:1000, Sigma) as the secondary antibody. The reaction was stopped adding 25 µL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. Standard curves were determined using purified lectin ($y = 0.0101x + 0.0025$, $R^2 = 0.998$).

2.13 Isoflavone determination by HPLC

Quantitative analysis of isoflavones was carried out according to the procedure of Berhow, 2002 (*26*). Approximately 250 mg defatted soybean flour were extracted in test tubes with 3.0 mL of dimethyl sulfoxide:methanol (1:4 v/v) placed in sealed containers and heated at 50˚ C for 18 hours. The extracts were centrifuged and the supernatants were filtered using 0.45 micron filters. For

isoflavone quantification, 20 µL aliquots of the extracts were injected into a Shimadzu (Columbia, MD) HPLC system (LC-10AT VP) equipped with a SPDM10A VP photodiode array detector an (CTO-10AS VP) oven column to maintain temperature at 40 ℃, all operating under the Class VP software. Isoflavone separation was carried out in a C18 reverse-phase column YMC - Pack ODS-AM, 250mm x 4.6mm and 5µm particle size (YMC Co, Ltd). The initial gradient conditions consisted of 100% H_2O containing 0.025% trifluoroacetic acid (TFA), and 0% acetonitrile, to 45% H_2O and 55% acetonitrile, over 25 min. with a flow rate of 1 mL/min. Isoflavones were detected at 260 nm and quantified by comparison with standard curves of genistin, daidzin and glycitin. The concentrations of the malonyl-glucosides and aglycones were calculated from standard curves of their corresponding *β*-glucosides, using the similarity of the molar extinction coefficients of malonyl-isoflavones and *β*-glucosides. Isoflavone concentrations were expressed in mg/100 g of defatted samples.

2.14 Saponin determination by HPLC

Saponins from the soybean flour were extracted with dimethylsulfoxide/methanol (1/1) solution at room temperature for 4 h, followed by a 15 min sonication at 50 ˚C and another 2 h extraction at room temperature. The extracts were then filtered through a 0.45 µm nylon filter. HPLC analysis was carried out on a Hewlett-Packard Series 1100 HPLC system equipped with an Inertsil ODS-3 reverse phase C-18 column (250 mm x 4.6 mm ID) and particle size of 5 µm, with a metaguard column (Varian) and a G1316A column oven. The system was controlled by HPChem Station version A.06.01. For saponin analysis, a linear water-acetonitrile gradient from 30% to 50% in 45 min was used, with 0.025% TFA added to both solvents. The flow rate was 1 mL/min and the effluent was monitored at 210 nm. Saponin concentrations were calculated by using standard curves prepared from a standardized mix of B group saponins prepared in the Peoria laboratory. The nanomolar extinction coefficient for Saponin I, was

used to quantitate the remaining B group saponins, the A group saponins, and the DMPP (1,l-dimethyl-4-phenylpiperzinium) conjugated B group saponins. Identification of saponin peaks was confirmed by comparison of standard and/or LC-MS analysis (*15*).

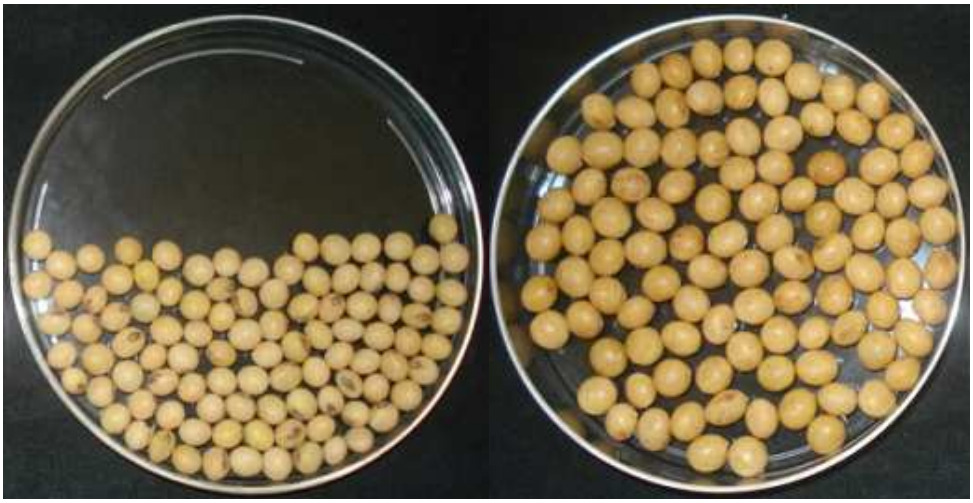
2.15 Statistical analysis

The data were submitted to analysis of variance by the SAS program (*27*), and the means of three replicates (unless otherwise stated) compared by the Tukey test, adopting the standard criterion of significance $p \leq 0.05$.

3. Results and discussion

3.1 Proximal composition

Cultivars BRS 133 and BRS 258 differed significantly in terms of size and weight, as it can be seen through Figure 1. The weights of 1000 seeds were 129.50 ± 0.12 and 227 ± 0.15 g, respectively.



BRS 133 **BRS 258**

Fig 1. Soy 100-bean samples of cultivars BRS 133 and 258

Proximal composition and the physicochemical characteristics of both cultivars are shown in **Table 1**. As expected the remarkable difference in protein concentration was reflected in their lipid and fiber contents, as was already reported by Vieira, Cabral & Oliveira de Paula (*28*) and Mandarino, Carrão-Panizzi & Oliveira (*29*). As pointed out by Morais (*30*), although all oligossacharides and disaccharides from soybean are fermentable, the 1 to 2% of raffinose and 3.5 to 4.5% of stachyose have an important bifidogenic role in the human intestine. In spite of the exact significance of soybean fiber in reducing the risk of colon cancer and cardiovascular diseases not being completely elucidated, the potential health benefits of this fraction should not be neglected. Soybean hulls contain about 87% total fiber, made up mainly of cellulose, hemicellulose, lignin and uronic acids (*31*). The values found in this study were similar to those reported by Toledo *et al*. (*32*) for various soybean cultivars.

3.2 Instrumental color of the flours

The values found for the L* parameters of the whole flours were 84.58 for cultivar BRS 133 and 81.37 for cultivar 258, respectively (**Table 1**). Such high values indicated that both cultivars could produce light-colored flours. The chrome parameters C*, in turn, indicated that cultivar BRS 258 had a higher degree of saturation (22.89), than the cultivar BRS 133. Considering the h parameter the values found were (89.41 and 87.49 for BRS 133 and BRS 258, respectively. It could be stated therefore that the flours of both cultivars are of a rather intense and light yellow color.

3.3 Particle size

The particle size analysis (**Table 1**) revealed that, despite the differing composition of both cultivars, both grains have similar matrix structures. Approximately 90% of the mass of the meals produced was retained within screens

28, 35 and 60 mesh. The remaining 10% present particle size was lower than 80 mesh.

Table 1. Chemical composition of the soybean cultivars BRS 133 and BRS 258, and physical characteristics of the whole flours.

Analysis[1]	Soybean cultivar	
	BRS 133	**BRS 258**
Percent composition (g/100g)		
Total Protein	37.36 ± 0.23 b	42.29 ± 0.16 a
Lipids	21.42 ± 0.22 b	22.74 ± 0.07 a
Ash	4.92 ± 0.08 a	4.83 ± 0.02 a
Carbohydrates (by difference)	36.30	30.14
Total Dietary Fiber	25.98 ± 0.02 a	25.80 ± 0.02 a
- Soluble Fiber	2.78 ± 0.12 a	2.87 ± 0.12 a
- Insoluble Fiber	23.20 ± 0.02 b	22.95 ± 0.02 b
Total Sugars	10.02 ± 0.03 a	4.03 ± 0.03 b
Starch	0.29 ± 0.01 a	0.31± 0.02 a
Metabolizable Energy (kcal·100g)	487.42	494.38
Color		
L* Lightness	84.58 ± 0.48 a	81.37 ± 0.07 b
C* Chrome	20.37 ± 0.11 b	22.89 ± 0.39 a
h* hue angle	89.41 ± 0.10 a	87.49 ± 0.13 b
Particle size (% retained in each screen)		
20 mesh (840 μm)	3.25 ± 0.01 a	3.57 ± 0.01 a
28 mesh (600 μm)	17.27 ± 0.01 a	17.72 ± 0.02 a
35 mesh (500 μm)	35.75 ± 0.01 a	34.85 ± 0.03 a
60 mesh (250 μm)	38.48 ± 0.02 b	40.27 ± 0.01 a
80 mesh (180 μm)	3.13 ± 0.04 a	3.33 ± 0.04 a
100 mesh (150 μm)	1.82 ± 0.02 a	0.23 ± 0.02 b
Bottom (< 150 μm)	0.31 ± 0.01 a	0.02 ± 0.00 b
pH		
pH at 25℃	6.60 ± 0.01 a	6.57 ± 0.03 a
Water Activity		
Water Activity at 25℃	0.66 ± 0.02 a	0.55 ± 0.00 b

[1] Means with different superscript letters in the same row are significantly different ($p < 0.05$).

3.4 Fatty acid composition

Fatty acid composition is shown in **Table 2.** We can observe high contents, about 80%, of unsaturated fatty acids, with linoleic acid (*cis* 18:2) being the predominant fatty acid (~56%), followed by oleic acid (~17%). The most abundant saturated fatty acid in both cultivars was palmitic acid (~11%). Between 1 to 3% of the oil was unsaponifiable material, such as steroids (stigmasterol, kaempesterol and sitosterol), tocopherols and provitamin-A carotenoids (*33*). The elevated iodine value, between 134 and 136, indicates the high degree of unsaturation.

Table 2. Fatty acid composition of the soybean cultivars BRS 133 and BRS 258.

Fatty acid[1]	Soybean cultivar	
	BRS 133	BRS 258
(C14:0) Myristic	0.09 ± 0.00 a	0.08 ± 0.00 a
(C16:0) Palmitic	11.78 ± 0.02 a	11.24 ± 0.01 a
(C16:1) Palmitoleic	0.09 ± 0.00 a	0.09 ± 0.00 a
(C18:0) Stearic	4.21 ± 0.13 a	3.56 ± 0.00 b
(C18:1 cis) Oleic	17.17 ± 0.05 b	20.25 ± 0.00 a
(C18:2 cis) Linoleic	56.01 ± 0.10 a	56.37 ± 0.01 a
(C18:3 cis) Linolenic	9.40 ± 0.05 a	7.37 ± 0.03 b
(C20:0) Araquidic	0.41 ± 0.00 a	0.35 ± 0.00 a
(C20:1) Gadoleic	0.16 ± 0.00 a	0.16 ± 0.00 a
(C22:0) Behenic	0.51 ± 0.01 a	0.38 ± 0.00 b
(C24:0) Lignoceric	0.17 ± 0.02 a	0.15 ± 0.00 a
Saturated FA	17.16	15.76
Monoinsaturated FA	17.42	20.50
Polyunsaturated FA	65.42	63.74
Calculated Iodine Index (g/100g)	136.54	134.52
Calculated Saponification Index	191	191

[1] Means with different superscript letters in the same row are significantly different ($p < 0.05$).

3.5 Amino acid composition

The total amino acid composition of a food protein, particularly of the essential amino acids, has classically been considered a measure of the biological adequacy of every food source**.** The total amino acids contents of both cultivars are shown in **Table 3.** These are compared to the official amino acid profile of an ideal protein as establishes by WHO-FAO-UNU (*34*). It could be observed that there were no significant differences between the two cultivars, with the exception of limiting methionine, which determined significantly different chemical scores for the two soybean cultivars.

The free amino acid composition of the two cultivars is presented in **Table 4.** Although the total free amino acids was essentially equal, cultivar BRS 133 showed a tendency to have a higher content of free amino acids than cultivar BRS 258, except for cysteine (high concentration), and glycine and glutamine concentration, no other single amino acid seemed to stand out in either of the two cultivars.

3.6 Minerals

Mineral composition is shown in **Table 5.** Cultivar BRS 258 appeared to have higher content of both macro and micro minerals as compared to BRS 133. The contents reported in our study are similar to those already observed by Mandarino, Carrão-Panizzi and Oliveira for soybeans produced at different locations in Brazil (*29*). Calcium and phosphorus have high relevance in human nutrition. Calcium bioavailability from soy milk (22.2%) has been estimated to be 90% of that from cow's milk. As far as phosphorus, main forms in soybean are phytic acid, inorganic phosphates, phospholipids and nucleic acids. Phytic acid may account for 50 to 70% and phospholipids about 15% of the total amount (*35*).

Table 3. Total amino acid composition of the whole flours of the soybean cultivars BRS 133 and BRS 258, compared with the WHO / FAO / UNU standard (2007).

Amino acid[1] (g/100g de protein)	Requirements* WHO / FAO / UNU			Soybean cultivar	
				BRS 133[1]	BRS 258[1]
	1-2	3-10			
Histidine (Hys)	-	-	1.5	2.63 ± 0.01 a	2.75 ± 0.01 a
Isoleucine (Ile)	-	-	3.0	4.44 ± 0.01 a	4.49 ± 0.03 a
Leucine (Leu)	-	-	5.9	7.57 ± 0.04 a	7.50 ± 0.01 a
Lysine (Lys)	5.2	4.8	4.5	6.16 ± 0.04 a	6.10 ± 0.01 a
Methionine (Met)	-	-	1.6	1.22 ± 0.01 a	1.01 ± 0.02 b
Cystina (Cys)	-	-	0.6	1.93 ± 0.02 a	1.67 ± 0.01 a
Phenylalaline (Phe)	-	-	-	4.82 ± 0.03 a	4.88 ± 0.03 a
Tyrosine (Tyr)	-	-	-	3.55 ±0.04 a	3.37 ± 0.00 a
Threonine (Thr)	2.7	2.5	2.3	3.93 ± 0.01a	3.68 ± 0.04 b
Tryptophan (Trp)	7.4	6.6	0.6	n.d.	n.d.
Valine (Val)	-	-	3.9	4.49 ± 0.03 a	4.31 ± 0.02 a
Total AAE[1]				40.74	39.76
	15.3	13.9	23.9		
Arginine	-	-	-	8.68 ± 0.01 b	9.42 ± 0.03 a
Alanine	-	-	-	4.30 ± 0.00 a	4.27 ± 0.01 a
Aspartic acid	-	-	-	11.61 ± 0.01 a	11.59 ± 0.04 a
Glutamic acid	-	-	-	18.54 ± 0.01 a	18.97 ± 0.02 a
Glycine	-	-	-	5.11 ± 0.01 a	4.95 ± 0.07 a
Proline	-	-	-	4.98 ± 0.00 a	4.99 ± 0.01 a
Serine	-	-	-	6.06 ± 0.02 a	6.04 ± 0.01 a
TOTAL				100	100
Sulfur amino acids (Met + Cys)	2.6	2.4	2.4	3.15	2.68
Aromatic (Phe + Tyr)	-	-	3.8	8.37	8.25
Chemical score				76	63

n.d.= not determined

[1] Means (two duplicates ± SE) with different superscript letters in the same row are significantly different ($p < 0.05$).

Source*: WHO, 2007.

Table 4. Free amino acid composition of the protein fraction of the whole flours of cultivars BRS 133 and BRS 258[1].

Free Amino Acids[2]	Soybean cultivar	
	BRS 133[2]	BRS 258[2]
Histidine	0.12 ± 0.00 a	0.11 ± 0.00 a
Isoleucine	0.10 ± 0.00 a	0.09 ± 0.00 b
Leucine	0.10 ± 0.00 a	0.09 ± 0.00 b
Lysine	0.12 ± 0.00 a	0.10 ± 0.00 b
Methionine	0.12 ± 0.00 a	0.10 ± 0.00 b
Cysteine	0.19 ± 0.00 a	0.16 ± 0.00 b
Phenylalanine	0.13 ± 0.00 a	0.11 ± 0.00 b
Tyrosine	0.14 ± 0.00 a	0.12 ± 0.00 b
Threonine	0.09 ± 0.00 a	0.08 ± 0.00 b
Tryptophan	0.16 ± 0.00 a	0.14 ± 0.00 b
Valine	0.09 ±0.00 a	0.08 ± 0.00 b
Arginine	0.13 ± 0.00 a	0.10 ± 0.00 b
Alanine	0.07 ± 0.00 a	0.05 ± 0.00 b
Aspartic Acid	0.07 ± 0.01 a	0.07 ± 0.00 a
Glutamic Acid	0.11 ± 0.01 a	0.09 ± 0.00 a
Glycine	0.06 ± 0.00 a	0.05 ± 0.00 b
Proline	0.09 ± 0.00 a	0.08 ± 0.00 b
Serine	0.08 ± 0.00 a	0.07 ± 0.00 b
Hydroxyproline	0.11 ± 0.00 a	0.09 ± 0.00 a
Asparagine	0.13 ± 0.00 a	0.11 ± 0.00 b
Glutamine	0.06 ± 0.01 a	0.05 ± 0.00 b
Taurine	0.09 ± 0.00 a	0.07 ± 0.00 b
TOTAL	2.36	2.01
Sulfur amino acids (Met + Cys)	0.31	0.26
Aromatic (Phe + Tyr)	0.27	0.23

[1] g per 100g of total protein, dry basis
[2] Means (two duplicates ± SE) with different superscript letters in the same row are significantly different ($p < 0.05$).

3.7 Bioactive compounds

The results for the functional compounds lunasin, BBI and lectin are reported in **Table 6**. The quantified lunasin was further identified and confirmed in a band (5.45 kDa) by Western blot analysis. Lunasin contents were within the ranges for different soybean genotypes reported by Gonzáles de Mejía et al. (*3*). Although there was no statistical difference in soluble protein concentration

between the two cultivars, the concentrations of lunasin, BBI and lectin in cultivar BRS 258 were statistically higher than in the low-protein BRS 133.

Table 5. Mean values of macro and microelements in the whole flours of soy cultivars BRS 133 and BRS 258[1].

Minerals[2]	Soybean cultivar	
	BRS 133[2]	BRS 258[2]
Macroelements		
Calcium	290.41 ± 1.47 b	335.37 ± 1.27 a
Phosphorus	524.40 ± 1.24 b	682.17 ± 0.23 a
Microelements		
Iron	22.30 ± 0.07 b	26.46 ± 0.25 a
Copper	2.88 ± 0.04 b	3.14 ± 0.06 a
Zinc	7.42 ± 0.16 b	8.27 ± 0.10 a

[1] Means (two replicates ± SE) with different superscript letters in the same row are significantly different ($p < 0.05$).
[2] mg per 100g, dry basis

Table 6. Bioactive compounds of soybean cultivars BRS 133 and BRS 258[1].

Analyses	Soybean cultivar	
	BRS 133	BRS 258
Soluble Protein (mg/g flour)	248.13 ± 2.21 a	244.19 ± 2.02 a
Bioactive Compounds (mg/g soluble protein)		
Lunasin	12.29 ± 0.54 b	14.78 ± 0.13 a
BBI	23.62 ± 0.36 b	28.11 ± 0.74 a
Lectin	16.96 ± 0.72 b	23.28 ± 0.14 a
Bioactive Compounds (mg/g flour)		
Lunasin	3.05 ± 0.14 b	3.61 ± 0.28 a
BBI	5.86 ± 0.33 b	6.86 ± 0.42 a
Lectin	4.21 ± 0.17 b	5.68 ± 0.03 a

[1] Means (three replicates ± SE) with different superscript letters in the same row are significantly different ($p < 0.05$).

3.8 Isoflavone content

The outstanding difference between the two cultivars was that cultivar BRS 133 exhibited a greater concentration of total isoflavones (390.00 mg/100 g of defatted flour), against 222.37 mg for BRS 258 (**Table 7**).

Research concerning soybean isoflavones has revealed their protective effect in health problems associated with menopause, cancer and cardiovascular diseases. Some other health benefits are under investigation (*1*). During processing, some losses and or shifting of the distribution profile of isoflavones may occur (*35*). The main isoflavones found in unprocessed soy flour, malonylgenistin, genistin, malonyldaidzin and daidzin, are converted into their aglycones and acetylglycosides forms. The concentration of aglycones, β-glucosides and malonylglucosides were 6.8 %, 22.8 % and 70.3 %, respectively for cultivar BRS 133, and 11.4 %, 19.7 % and 68.8 %, respectively for cultivar BRS 258.

Table 7. Mean isoflavone concentrations of soybean cultivar BRS 133 and BRS 258[1]

Isoflavones[2]	Soybean cultivar	
	BRS 133	**BRS 258**
Aglicones		
Daidzein	10.98 ± 0.10 a	7.69 ± 0.04 b
Genistein	14.40 ± 0.02 b	15.41 ± 0.05 a
Glycitein	1.31 ± 0.05 b	2.30 ± 0.06 a
β-glucosides		
Daidzin	42.46 ± 0.02 a	14.71 ± 0.04 b
Genistin	36.12 ± 0.10 a	23.09 ± 0.06 b
Glycitin	10.40 ± 0.04 a	6.02 ± 0.02 b
Acetylglucosides		
Acetyldaidzin	0	0
Acetylgenistin	0	0
Acetylglycitin	0	0
Malonylglucosides		
Malonyldaidzin	131.62 ± 0.06 a	57.56 ± 0.03 b
Malonylgenistin	100.75 ± 0.10 a	72.96 ± 0.02 b
Malonylglycitin	41.96 ± 0.04 a	22.64 ± 0.04 b
Total aglycones	26.69 ± 0.07 a	25.40 ± 0.05 b
Total isoflavones	390.00 ± 0.10 a	222.37 ± 0.09 b

[1] mg/100g defatted flour, dry basis.
[2]Means (two duplicates ± SE) with different superscript letters in the same row are significantly different ($p < 0.05$).

3.9 Saponin content

As was noticed with the isoflavones, cultivar BRS 133 exhibited high contents of total saponins (9.7 mg/100 g of defatted soy flour), as opposed to 7.4 found in the cultivar BRS 258 (**Table 8**). On the basis of their aglycone structures, the saponins present in the mature bean have been divided into group B and group A soyasaponins (*15*).

Group B soyasaponins appear to exist in the intact seed tissue as conjugates of 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP), at the 22 hydroxyl position (*36*). The DDMP conjugates are relatively labile and are easily degraded, most likely resulting in the formation of the non-DDMP group B soyasaponins. The other various forms of the group B soyasaponins arise from alternate sugars attached to the 3-hydroxyl position of the aglycone. The group A soyasaponins are didesmosidic with alternate sugar compositions in both sets of oligosaccharides attached to the aglycone at the 3- and 21-hydroxyl positions (*37*).

Table 8. Saponin concentration in the soybean cultivars BRS 133 and BRS 258[1].

SAPONINS (mg/g flour)	Cultivar	
	BRS 133	BRS 258
B group		
Soyasaponis I	1.67 ± 0.04 a	1.26 ± 0.00 b
Soyasaponis II	0.23 ± 0.01 a	0.22 ± 0.00 b
Soyasaponis III	0.74 ± 0.01 a	0.27 ± 0.01 b
Soyasaponis IV	0.13 ± 0.01 a	0.10 ± 0.01 b
Soyasaponis V	0.29 ± 0.01 a	0.21 ± 0.00 b
Soyasaponis βg	2.25 ± 0.07 a	1.69 ± 0.07 b
Soyasaponis βa	0.24 ± 0.02 a	0.12 ± 0.01 b
Soyasaponis γg	0.94 ± 0.03 a	0.57 ± 0.03 b
Soyasaponis γa	0.14 ± 0.01 a	0.09 ± 0.00 b
Soyasaponis αg	0.55 ± 0.02 a	0.42 ± 0.01 b
A group		
Soyasaponis aA1	2.30 ± 0.03 a	2.29 ± 0.10 a
Soyasaponis aA2	0.23 ± 0.01 a	0.14 ± 0.01 b
Soyasaponis aA7	0.04 ± 0.01 b	0.06 ± 0.00 a
Total Soyasaponis	9.75 ± 0.18 a	7.44 ± 0.05 b

[1] Means with different superscript letters in the same row are significantly different ($p < 0.05$).

4. Conclusions

The comprehensive chemical characterization data gathered for these two different Brazilian soybean cultivars permit to conclude that although their composition fall within a typical range of soybean nutrients, a distinctive pattern emerges for some nutrients and bioactive compounds with respect to the protein content. The higher protein content of cultivar BRS 258 seemed to have occurred mostly at the expense of the carbohydrate fraction of the grain.

Both cultivars exhibited normal total and free amino acid composition in spite of the fact that the low-protein soybean cultivar BRS 133 had a higher amino acid score (76) than the high-protein soybean cultivar BRS 258 (63), apparently due to some unidentified storage protein fraction poor in methionine, which may be responsible for the extra protein filling of the protein richer grain. Since both cultivars were produced in Paraná State - Brazil and under equivalent cultivation techniques, the contents of minerals, ranging from 9 to 30% higher in the cultivar BRS 258 in relation to cultivar BRS 133, could be directly related to the protein deposition in the seed. Similar physiological mechanisms could explain higher contents of the proteinaceous components lunasin, Bowman-Birk inhibitor and lectin found in cultivar BRS 258.

On the other hand, the nearly 75.4% higher total isoflavone content found in cultivar BRS 133, with 5.08% more aglycones, makes it more appropiate for the formulation of foods with health benefit claims.

Analogously, total soyasaponins were about 31.04% higher in this cultivar as compared to BRS 258 (protein-rich cultivar).

5. Acknowledgments

The authors wish to thank CAPES-PEC PG for the scholarship granted to Luz Maria Paucar-Menacho and Mr. Rodolfo Rohr Neto (SoSoja do Brasil Ltda.) and Mr. Kenji S. Narumiya (Sun Foods-Brasil) for the financial support. The donation of the soybean cultivars BRS 133 and 258 by Embrapa Soybean and Embrapa Technology Transfer, Brazil, and the FAEPEX for the grant from the Foundation for Teaching, Research and Extension (Unicamp) are also acknowledged. The authors are also grateful to MSc. Rosa Helena Aguiar, Mrs. Carla Greghi and Éder Muller Risso for their kind technical assistance, and to Dr. Patricia Luna Pizarro, of the National University of Jujuy, Argentina for her laboratorial support.

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Capítulo 3

Optimization of germination time and temperature on the concentration of bioactive compounds in Braziliam soybean cultivar BRS 133 using response surface methodology

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This paper was submited to Food Chemistry on Jan 05th, 2009

Capítulo 3: Optimization of germination time and temperature on the concentration of bioactive compounds in Braziliam soybean cultivar BRS 133 using response surface methodology

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Abstract

The consumption of soybeans and soybean products has increased considerably in the last few years, due to the functional properties accounted to the presence of bioactive compounds such as lunasin, BBI, lectin, saponins and isoflavones which bring health benefits to consumers. The objective of this work was to influence of the germination process of soybean seeds from cultivar BRS 133 on this bioactive compounds. Germination was carried out in a germination chamber with paper; germinated samples were frozen at -30℃, freeze-dri ed and milled to produce germinated whole soybean flour. Isoflavone and saponin determinations were analyzed by HPLC. Lunasin, BBI and lectin were analyzed by ELISA and lunasin identity through Western Blot assay. The effects of the variations in germination time and temperature were analyzed using the Response Surface Methodology (RSM), with a 2^2 central composite rotational design. The independent variables that were studied were time and temperature. The germination conditions of soybean BRS 133 modified the contentrations of bioactive compounds within the ranges studied and it increased content of lunasin, isoflavone aglycones and soyasaponins and decreased the content of BBI, lectin and lipoxygenase. Optimal increases in the concentrations of isoflavone aglycones (daidzein and genistein) were observed in combination of 63 h germination time and 30 ℃. A significant increase in the content of soyasaponins was observed with the combination of 42 h germination time and 25 ℃.

KEYWORDS: Soybean-BRS 133, germination, lunasin, Bowman-Birk inhibitor, lectin, isoflavones and saponins.

1. Introduction

The direct use of soybeans in human foods is limited due to the presence of several anti-nutritional factors. The majority of processed soybean products are derived from dry mature soybeans. However, the development of products from germinated soybean is another way to further increase the versatility and utilization of soybeans. Germination has been identified as an inexpensive and effective technology for improving the nutritional quality of soybeans (Bau, Villaume, Nicolas & Méjean, 1997). Effects of germination conditions (temperature, light, moisture, and germination time) on bioative compounds vary greatly with the plant species, seed varieties or cultivars (Edwards, 1934; Wuebker, Mullen & Hoelher, 2001; Gloria, Tavarez-Neto & Labanca, 2005). Soybean is a complex matrix containing several bioactive compounds, including lunasin, Bowman Birk Inhibitor (BBI), lectins, isoflavones, soyasaponins, and other soy proteins and bioactive peptides with cancer-preventive properties. Lunasin is a novel, cancer-preventive peptide whose efficacy against chemical carcinogens and oncogenes has been demonstrated in mammalian cells and in a skin cancer mouse model (de Lumen, 2005). Lunasin and BBI are bioactive soy peptides that have been shown to be effective suppressors of carcinogenesis *in vitro* and *in vivo* model systems (Park, Jeong & de Lumen, 2007). Lectins are glycoproteins that selectively bind carbohydrates. Several lectins have been found to possess anticancer properties in vitro, in vivo, and in human case studies. They are used as therapeutic agents, preferentially binding to cancer cell membranes or their receptors, causing cytotoxicity, apoptosis, and inhibition of tumor growth (Gonzáles de Mejía & Prisecaru, 2005).

Soybean seeds are a relatively rich source of lipoxigenases, which are an important factor in the generation of odor and off-flavor compounds from lipids and also deteriorate palatability. Short periods of germination (72 h) can substantially improve odor and flavor scores of full fat soybean flour because lipoxygenase activity is reduced during the germination process, hence non-defatted flour

germinated seed would have a more stable shelf-life (Suberbie, Mendizabal & Mendizabal , 1981).

The major soy isoflavones *β*-glucosides are genistin and daidzin, and glycitin and their malonyl and acetil conjugates at the C-6 position of the glucose group (Anderson & Wolf, 1995). Soybean products may also contain small to large amounts of the aglycone forms: genistein, daidzein and glycitein. Saponins are plant triterpenoid glycosides generally derived from sugar substituted forms of sapogenol A and sapogenol B. Germination induces a substantial increase in the concentration of a variety of estrogenic compounds and almost all phytosterols, particulary *β*-sitosterol (Bau, Villaume & Méjean, 2000).

The objective of this study was to determine the influence of various germination conditions on the concentration of bioactive compounds in Brazilian soybean cultivar BRS 133 using RSM analysis. Therefore, this study involved evaluating the optimum conditions of germination time and temperature on the concentration of soluble protein, lunasin, BBI, lectin, saponins and isoflavones.

2. Materials and Methods

2.1 Material

Soybean cultivar BRS 133, with weight of 129 g per 1000 seeds, was developed as part of the breeding program of Embrapa Soybean, Brazil**.** This cultivar was selected because of its low level of protein and its high levels of isoflavones (EMBRAPA, 2008; Mandarino, Carrão-Panizzi, & Crancianinov, 2006). Soybeans seeds of BRS 133 (8.39% moisture) were cleaned with sodium hypochlorite (100 mg/kg) for 10 min, and then rinsed three times with distilled water and kept at room temperature for 8 h. Germination was carried out in germination chambers using paper in trays containing 500 g seeds each. Germinated seeds were then frozen at –30 ℃ for 4 hours, freeze-drie d, and milled to produce germinated soybean obtained in a refrigerated hammer mill, model 680 from

Marconi (Piracicaba, Brazil), and the powders stored at 7℃, conditioned in air-tight glass.

Immunoaffinity purified lunasin (98%) from soy and rabbit polyclonal antibody against the lunasin epitope –EKHIMEKIQGRGDDDDD were provided by Dr. Ben O. de Lumen from the University of California at Berkeley. Purified A and B group soy saponins were prepared in the Peoria laboratory (USDA) (Brehow, Kong e Duval, 2006).

The primary polyclonal antibody that is specific for lectin from soybean was provided by Dr. Theodore Hymowitz from the Department of Crop Sciences, University of Illinois at Urbana-Champaign. The lectin anti-serum was obtained by immunizing young male New Zealand white rabbits with a subcutaneous injection of 5 mL emulsion containing 5 mg of pure lectin, 1 mL of distillated water and 1 mL of Freund's complete adjuvant. Six weeks after the first immunization, rabbits showing response to the antibodies (measured 20 days after the first injection) were injected again with a similar dose and bled two weeks later (Orf, 1979).

2.2 Protein extraction

Fifty mg of soybean flour and 1 mL of extracting buffer (0.05 M Tris-HCl buffer, pH 8.2) were placed in an Eppendorf tube. After mixing, the samples were sonicated in an ultrasonic bath (Branson Ultrsonic Corporation, Danbury, CT) for 70 min, mixing every 10 min to avoid settling, at 40 ℃ using a recirculation bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, NH). The samples were centrifuged at 20,000 g for 40 min, at 8 ℃, in an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY), and the supernatant was decanted to a new Eppendorf tube.

2.3 Determination of soluble protein concentration by DC assay

The protein concentration was determined using the Bio-Rad *DC* Microplate Assay Protocol (Bio-Rad Laboratories, Hercules, CA). Briefly, 5 µL of samples

(1:20 dilution) were placed in a 96-well plate and treated with 25 µL of Bio-Rad A (alkaline copper tartrate solution) and 200 µL of Bio-Rad reagent B (dilute Folin reagent) (Bio-Rad Laboratories, Hercules, CA). The plate was gently agitated and incubated for 15 min at room temperature. After incubation, the absorbance was measured at 630 nm. The protein concentration was calculated using pure bovine serum albumin standard curve (y = 0.0002x - 0.0021, R^2 = 0.997).

2.4 Enzyme-linked immunosorbent assay (ELISA) for lunasin and BBI

Lunasin concentration in soy flour from germinated seeds was determined by ELISA (González de Mejía, Vasconez, de Lumen & Nelson, 2004) with the following modifications. Samples of 100 µL of protein extracts (1:5000 dilution) were placed in a 96-well plate and stored for 14 h. Lunasin mouse monoclonal antibody (1:4000 dilution) was used as the primary antibody and anti-mouse IgG alkaline phosphatase conjugate (1:7000) (Sigma Chem, St. Louis, MO) as the secondary antibody. The reaction was stopped adding 25 µL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. A similar procedure was used for BBI analysis. Samples of 100 µL of protein extracts (1:10000 dilution) were placed in a 96-well plate, except that BBI mouse monoclonal antibody (1:1000 dilution) (Agdia, Inc., Elkhart, IN) was used as the primary antibody and anti-mouse alkaline phosphatase (AP) conjugated IgG (1:2000) as the secondary antibody. Standard curves were determined using purified lunasin (y = 0.0054x + 0.001, R^2 = 0.993) and purified BBI (y = 0.0108x + 0.0465, R^2 = 0.998).

2.5 Enzyme-linked immunosorbent assay (ELISA) for lectin

Lectin concentration in soy flour from germinated seeds was determined by ELISA (Vasconez–Costa, 2004) with the following modifications. One hundred microliters (100 µL) of protein extracts (1:10000 dilution) was placed in a 96-well plate. Lectin mouse polyclonal antibody (1:500 dilution) was used as the primary antibody, and anti-rabbit IgG alkaline phosphatase conjugate (1:1000, Sigma) as

the secondary antibody. The reaction was stopped adding 25 µL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. Standard curves were determined using purified lectin (y = 0.0101x + 0.0025, R^2 = 0.998).

2.6 Gel electrophoresis

To the supernatant of each protein extract (20 µL) were added 20 µL of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with 5% 2-mercaptoethanol in Eppendorf tubes which were then heated at 100 ˚C for 3 min. The samples (20 µL) and the standard (5 µL) were loaded in the wells of the gel. The gel was run in a Mini Protean-3 cell (Bio-Rad, Laboratories) using 10-20% gradient Tris-Glycine SDS buffer as then running buffer. The power was set at 400 mA (200 V) constant for 30 min. Gels were fixed with peptide fixing solution for 30 min in methanol/acetic acid/water (10:40:50) and were stained with Bio Safe Coomassie G = 250 (Bio-Rad Laboratories) overnight and then destained with a 10% solution of acetic acid. Gels were read in a Kodak Image Station 440 CF, where the respective molecular masses and band intensities were recorded. Amino acid sequences of major soy proteins were retrieved from UniProtKB/Swiss-Prot Release 54.1 of 21-Aug-2007. The theoretical molecular weight of each protein was calculated from the amino acid sequence with the ProtParam program (http://ca.expasy.org/tools/protparam.html). Identification of the lipoxygenase band (92.9 kDa) was confirmed by comparing the theoretical molecular weight with the experimental data.

2.7 Isoflavone content determination by HPLC

Quantitative analysis of isoflavones was carried out following the procedures proposed by Berhow, (2002). Approximately 250 mg defatted soybean flour was extracted in test tubes with 3.0 mL of dimethyl sulfoxide:methanol (1:4 v/v), placed in sealed containers and heated at 50 ℃ for 18 h. The extracts were centrifuged and the supernatants were filtered using 0.45 micron filters. For isoflavone

quantification, 20 µL aliquots of the extracts were injected into a Shimadzu (Columbia, MD) HPLC system (LC-10AT VP pumps) equipped with a SPDM10A VP photodiode array detector an (CTO-10AS VP) oven column to maintain temperature at 40 ℃, all operating under Class VP software. Isoflavone separation was carried out in a C18 reverse-phase column YMC – Pack ODS-AM, 250 mm x 4.6 mm, 5 *µ*m particle size (YMC Co, Ltd.). The initial gradient conditions consisted of 100% H_2O containing 0.025% trifluoroacetic acid (TFA), and 0% acetonitrile, to 45% H_2O and 55% acetonitrile, over 25 min. with a flow rate of 1 mL/min. Isoflavones were detected at 260 nm and quantified by comparison with standard curves for genistin, daidzin and glycitin. The concentrations of the malonyl-glucosides and the aglycones were calculated from standard curves of their corresponding *β*-glucosides, using the similarity of the molar extinction coefficients of malonyl-isoflavones and *β*-glucosides. Isoflavone concentrations were expressed in mg/100 g of defatted samples.

2.8 Saponin content determination by HPLC

Saponins from the germinated soybean flour were extracted with dimethylsulfoxide/methanol (1/1) solution at room temperature for 4 h, followed by a 15 min sonication at 50 ˚C and another 2 h extraction at room temperature. The extracts were then filtered through a 0.45 *µ*m nylon filter. HPLC analysis was carried out in a Hewlett-Packard Series 1100 HPLC system equipped with an Inertsil ODS-3 reverse phase C-18 column (250 mm x 4.6 mm ID) and particles size of 5 µm, with a metaguard column (Varian) and a G1316A column oven. The system was controlled by HPChem Station version A.06.01. For saponin analysis, a linear water-acetonitrile gradient from 30% to 50% in 45 min was used, with 0.025% TFA added to both solvents. The flow rate was 1 mL/min and the effluent was monitored at 210 nm. Saponin concentrations were calculated by using standard curves prepared from a standardized mix of B group saponins prepared in the Peoria laboratory. The nanomolar extinction coefficient for soyasaponin I, was used to quantitate the remaining B group saponins, the A group saponins, and

the DMPP (1,l-dimethyl-4-phenylpiperzinium) conjugated B group saponins. Identification of saponins peaks was confirmed by comparison of standard and/or LC-MS analysis (Berhow, Kong & Duval, 2006).

2.9 Experimental design

Variation effects in germination time and temperature were analyzed using the Response Surface Methodology (RSM), with a 2^2 central composite rotational design. The independent variables studied were: germination time (12, 21, 42, 63 and 72 h) and germination temperature (18, 20, 25, 30 and 32 ℃). Real and coded levels for these variables are given in **Table 1**.

Table 1. Real and coded levels of the independent variables used in the experiments with BRS 133 soybean seed.

Independent variables		Levels				
Coded	Real	-α	-1	0	+1	+ α
X_1	Germination time (h)	12	21	42	63	72
X_2	Germination temperature (℃)	18	20	25	30	32

±|α|=1.41.

2.10 Statistical analysis

Statistica 5.0 (Statsoft, USA) was used to determine the effects of the independent variables, calculate regression coefficients, carry out analysis of variance (ANOVA) and build the response surfaces, at a 5% significance level.
The following second order polynomial model was fitted to the data:

$$Y= \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \qquad (1)$$

Where **Y** is the response variable, $\mathbf{X_1}$ and $\mathbf{X_2}$ are the coded process variables and **βn** are the regression coefficients. A stepwise methodology was followed to determine the significant terms in Eq. 1.

3. Results and Discussion

Lunasin, BBI, lectin and soluble protein concentrations in non-germinated freeze-dried soybean are presented in Table 2. The experimental responses in terms of soluble protein (SP) (mg/g flour), for lunasin (mg/g SP), BBI (mg/g SP), lectin (mg/g SP) and lipoxygenase (%) in germinated freeze-dried soybean flou are presented in Table 3. The observed values of soluble protein, lunasin, BBI and lectin in soy flour with different combinations of germination time and temperature are summarized in Table 4.

Table 2. Lunasin, BBI, Lectin, total isoflavone and total soyasaponins in non-germinated freeze-dried BRS 133 soybean flour.

Components	(mg/g SP)	(mg/g Flour)
Lunasin	12.29 ± 0.54	3.05 ± 0.13
BBI	23.62 ± 0.36	5.86 ± 0.34
Lectin	16.96 ± 0.72	4.21 ± 0.18
Soluble Protein	-	248.13 ± 2.21

[1] Means with different superscript letters in the same row are significantly different ($p < 0.05$).

Table 3. Observed response values with diferents combinations of germination time and germination temperature for BRS 133.

Coded level			Response values				
Exp	X_1 (h)	X_2 (℃)	Soluble Protein (SP) (mg/g flour)	Lunasin (mg/g SP)	BBI (mg/g SP)	Lectin (mg/g SP)	Lipoxygenase (%)
1	-1 (21)	-1 (20)	298.61	11.20	28.29	12.72	9.38
2	+1(63)	-1 (20)	192.07	19.58	21.54	10.64	6.69
3	-1 (21)	+1(30)	201.85	18.54	27.08	12.74	8.85
4	+1 (63)	+1(30)	216.74	10.81	34.94	8.50	4.02
5	-α (12)	0 (25)	282.27	17.54	27.31	12.91	7.44
6	+α (72)	0 (25)	211.35	13.22	28.03	11.02	4.17
7	0 (42)	-α (18)	305.17	12.52	28.40	11.87	8.06
8	0 (42)	+α (32)	184.43	10.54	31.25	12.45	6.81
9	0 (42)	0 (25)	208.36	21.20	28.70	7.62	4.02
10	0 (42)	0 (25)	208.52	21.01	28.39	7.30	4.01
11	0 (42)	0 (25)	208.36	21.03	28.76	7.28	4.00

X_1= Germination time
X_2= Germination temperature
SP=Soluble protein
BBI= Bowman Birk inhibitor

Table 4. Observed values for lunasin, BBI and lectin in soy flour with diferents combinations of germination time and germination temperature for BRS 133.

Exp.	X_1 (h)	X_2 (℃)	Lunasin (mg /g flour)	BBI (mg /g flour)	Lectin (mg /g flour)
1	- 1 (21)	- 1 (20)	4.33 a	7.97 b	3.58 a
2	+1(63)	- 1 (20)	3.76 c	4.14 d	2.04 c
3	- 1 (21)	+ 1(30)	3.73 c	5.47 c	2.57 b
4	+1 (63)	+ 1(30)	2.34 e	7.57 b	1.40 d
5	-α (12)	0 (25)	4.95 a	7.71 b	3.64 a
6	+α (72)	0 (25)	2.80 d	5.59 c	2.33 bc
7	0 (42)	-α (18)	3.82 c	8.67 a	3.62 a
8	0 (42)	+α (32)	1.94 e	5.76 c	2.30 bc
9	0 (42)	0 (25)	4.42 b	5.98 c	1.52 d
10	0 (42)	0 (25)	4.38 b	5.92 c	1.52 d
11	0 (42)	0 (25)	4.38 b	5.99 c	1.52 d

[1] Means with different superscript letters in the same colum are significantly different ($p < 0.05$).

3.1 Soluble protein content in germinated soy flour

The soluble protein (SP) concentration in the protein extracts from the flour obtained from germinated soybean seeds varied from 184.43 mg/g to 305.17 mg/g. The regression model for this parameter was statistically significant ($p < 0.05$) with $R^2 = 0.90$ which indicates a good adjustment of the model to the experimental data. In this case, the non-significant interaction terms could be removed to make the regression equation simple with an $R^2 = 0.81$. The 2nd order adjusted model for soluble protein concentration is presented in Equation (2) and the response surface in **Figure 1A.**

$$\text{Soluble protein (mg/g flour)} = \mathbf{228.88 - 23.99\, x_1 - 30.35\, x_2 + 30.35\, x_1 x_2} \quad (2)$$

High values of SP were observed in the ranges from 12 h (-α) to 21 h (-1) of germination time and 18 ℃ (- α) to 20 °C (-1) germination temperature. As cultivar BRS 133 its low level of total protein (37.36%), maintaining the germination time constant at 21 h (-1) (comparing Exp. 1 and 3), an increse in germination

temperature from 20 ℃ (-1) to 30℃ (+1) promoted a decrease of 32.40% in soluble protein concentration.

3.2 Lunasin content and identity in the protein extract

Identification of the lunasin band (5.45 KDa) was confirmed by Western blot analysis. The results for lunasin were similar to those reported for different soybean genotypes by Gonzales de Mejia, Vásconez, De Lumen and Nelson, (2004) (**Figures 2A and 3A**). The lunasin concentration in the protein extracts from the flour obtained from germinated soybean flour varied from 10.54 to 21.20 mg/g SP. The regression coefficient for the complete model was 0.95. In this case, the non-significant interaction term could be removed to make the regression equation a 2nd order adjusted model for lunasin concentration with an R^2 = 0.91. This is presented in Equation (3) and the response surface in **Figure 1B**.

$$\text{Lunasin (mg/g SP)} = \mathbf{21.08 - 2.45x_1^2 - 4.38\ x_2^2 - 4.03\ x_1x_2} \quad (3)$$

High values of lunasin were observed at 21 h (-1) than 63 h (+1) of germination time and 20 ^{o} C (-1) to 30 ^{o} C (+1) of germination temperature. The optimal condition was exactly the central point (0,0) with 42 h germination time at 25℃. In this case, germination process contributed to an increase in lunasin levels from 12.29 mg/g SP in the non-germinated soybean flour to 21 mg/g SP in germinated soybean flour, resulting in an increase of up to 73.62% in this bioactive compound.

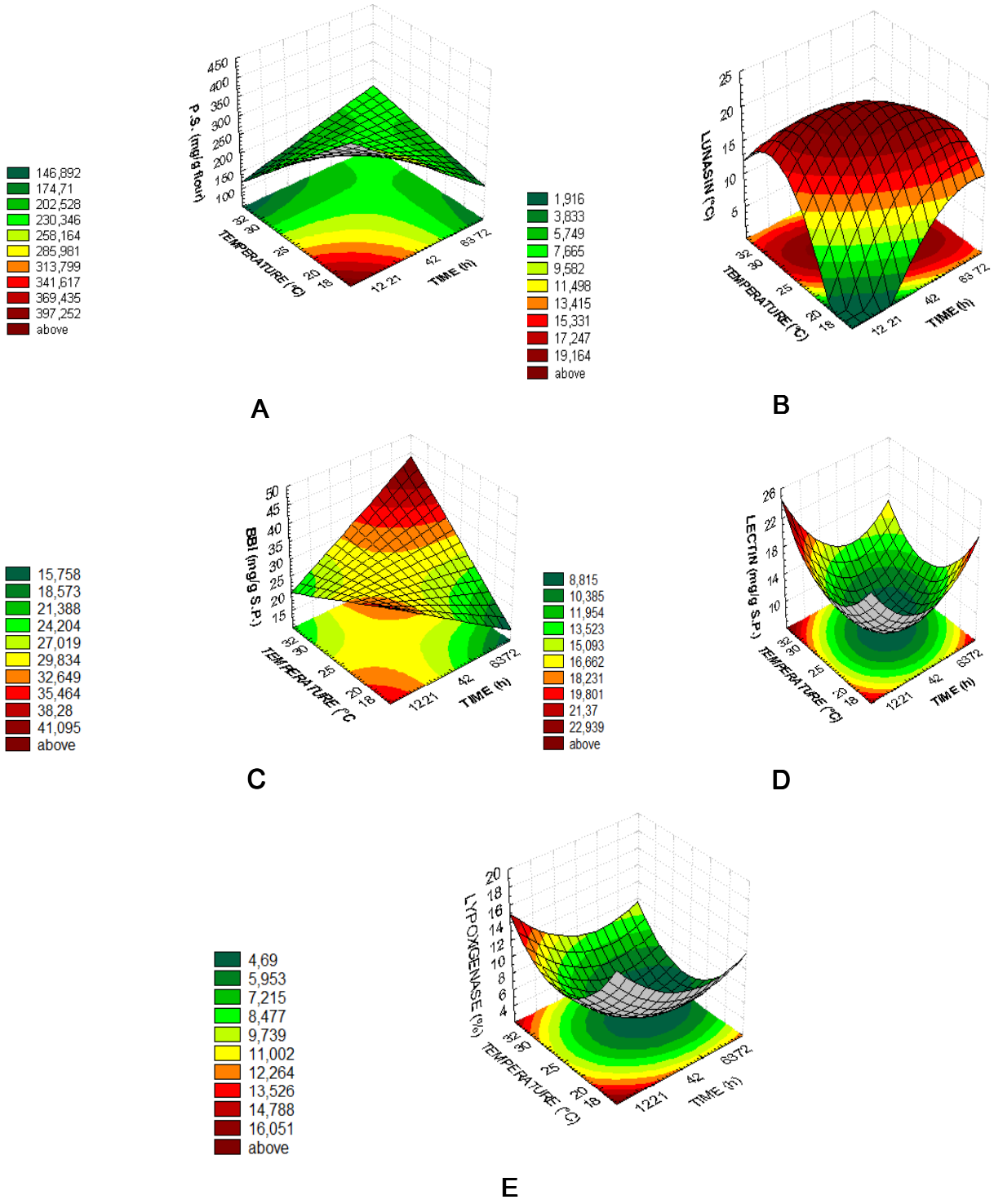


Figura 1. Response surfaces for compounds in soybean seed BRS 133 germinated flour showing time versus temperature. (**A**) Soluble protein. (**B**) Lunasin. (**C**) BBI. (**D**) Lectin. (**E**) Lipoxygenase.

3.3 Bowman Birk inhibitor content in protein extracts

The BBI concentration in the protein extracts of the flour obtained from germinated soybean seeds varied from 21.54 to 34.94 mg/g SP. The regression coefficient for the complete model was 0.90. In this case, the non-significant interaction terms could be removed to obtain an adjusted model for BBI contentration with an R^2 = 0.85, presented in Equation (4) and the response surface in **Figure 1C**.

$$\text{BBI (mg/g S.P.)} = \mathbf{28.43 - 2.03\ x_1 + 3.65\ x_1\ x_2} \qquad (4)$$

Lower values of BBI concentration in SP were observed at higher germination times [63 h (+1) to 72 h (+α)] and lower germination temperatures [18 ℃ (- α) to 20 ℃ (-1)] or lower germination time [12h (- α) to 21 h (-1)] and higher germination temperatures [30 ℃ (+1) to 32 ℃ (+ α)]. In this case, BBI concentration decreased only in Exp 2 (63 h of germination time at 20℃), a bout 8.8% in relation to the non-germinated soybean flour. Germination degrades trypsin inhibitor slowly in the beginning (Bau, Villaume, Nicolas & Méjean, 1997). Collins & Sanders (1976) found that a 24 h soaking process had only a slight effect at most on altering BBI of soybean, after 24 h soaking and a 3-day germination, BBI decreased only about 13% for Kanrich variety, 4% for Soylima variety and 8% for Dare variety.

3.4 Lectin content in protein extracts

The lectin concentration in the non-germinated freeze-dried soybean flour was 16.96 mg/g SP. Germination resulted in decreased lectin concentration in the protein extracts of the germinated flour, which varied, from 6.48 to 12.74 mg/g SP. The regression coefficient for the complete model was 0.92; but in this case, the non-significant terms could be removed to make the regression equation simple with an R^2 = 0.89. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Equation (5) and the response surface in **Figure 1D**.

$$\text{Lectin (mg/g S.P.)} = \mathbf{7.40 - 1.12x_1 + 2.05\ x_1^2 + 2.15\ x_2^2} \qquad (5)$$

The lowest values of lectin concentration in SP were observed for germination temperatures ranging from 20 °C (0) to 30°C (+1) and with 42 h (0) to 63 h (+1) of germination time. This fact should be an important effect of germination improving the biological and nutritional value of germinated soybeans and its utilization in human foods and animal feed (Bau, Villaume, Nicolas, Méjean, 1997). The optimal condition was the central point (0,0), with 42 h germination time at 25°C. In this case, the germination process contributed to a decrease in lectin levels from 16.96 mg/g SP in the non-germinated soybean flour to 7 mg/g SP in germinated soybean flour, resulting in a decrease of 55.07% in this bioactive compound.

3.5 Lipoxygenase concentration (%)

The identification of the lipoxygenase band (92.9 KDa) was confirmed by comparing the theoretical molecular weight with the experimental data (**Table 5**) and it is shown in **Figures 2B** and **3B**. The lipoxygenase concentration of the germinated soybean flour varied from 4.02 to 9.38 %, while the lipoxygenase contentration of the non-germinated freeze-dried soybean flour was 13.31 %. The regression model for this parameter was statistically significant ($p < 0.05$) and had an R^2 = 0.95. The 2nd order adjusted model (R^2 = 0.93) for lipoxygenase concentration is presented in Equation (6) and the response surface in **Figure 1E**.

$$\text{Lipoxygenase (\%)} = \mathbf{4.01 - 1.52x_1 + 1.05\ x_1^2 - 0.62\ x_2^2 + 1.86\ x_1x_2} \qquad (6)$$

Lower values of lipoxygenase content in SP were observed from 25 °C (0) to 30 °C (+1) of germination temperature and higher germination times [42 h (0) to 72 h (+α)]. The optimal condition was the central point (0,0), with 42 h germination time at 25°C. In this case, germination process contributed to a decrease in lipoxygenase content from 13.31% in the non-germinated soybean flour to 4% in germinated soybean flour, resulting in a decrease of 69.92%. Germination caused reduction in the level of specific activity of lypoxygenase 1 (Bordingnon, Oliveira &

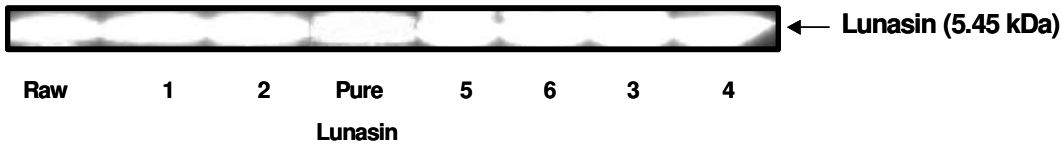
Mandarino, 1995). Commercial full-fat soy flour has no lipoxigenase activity and the stability of its lipid composition is constant (Suberbie, Mendizábal & Mendizábal, 1981).

Table 5. Calculated molecular weights of major soy proteins.[1]

	Name	Accession number	Number of amino acid	Molecular weight (Da)
Lipoxygenase	1			89,100.0
Lipoxygenase	2 and 3			92,900.0
	α´subunit	gi|9967361	554	65,142.6
β-conglycinin	α subunit	gi| 9967357	543	63,164.8
	β subunit	gi| 9967359	416	47,975.7
	G1 precursor	**P04776**	495	55,706.3
	A1 a chain	CHAIN_20-306	287	32,646.9
	Bx chain	CHAIN_311-490	180	19,955.5
	G2 precursor	**P04405**	485	54,390.7
	A2 chain	CHAIN_19-296	278	31,622.8
	B1a chain	CHAIN_301-480	180	19,773.2
glycinin	**G3 precursor**	**P11828**	481	54,241.7
	A chain	CHAIN_22-296	275	31,483.7
	B chain	CHAIN_297-476	180	19,911.4
	G4 precursor	**P02858**	562	63,587.1
	A5 chain	CHAIN_24-120	97	10,540.8
	A4 chain	CHAIN_121-377	257	29,953.9
	B3 chain	CHAIN_378-562	186	20,743.5
	G5 precursor	**P04347**	516	57,956.1
	A3 chain	CHAIN_25-344	320	36,392.4
	B4 chain	CHAIN_345-516	172	19,049.5

[1] Amino acid sequences of major soy proteins were retrieved from UniProtKB/Swiss-Prot Release 54.1 of 21-Aug-2007, and the theoretical molecular weight of each protein was calculated using the ProtParam program (http://ca.expasy.org/tools/protparam.html).

A



B

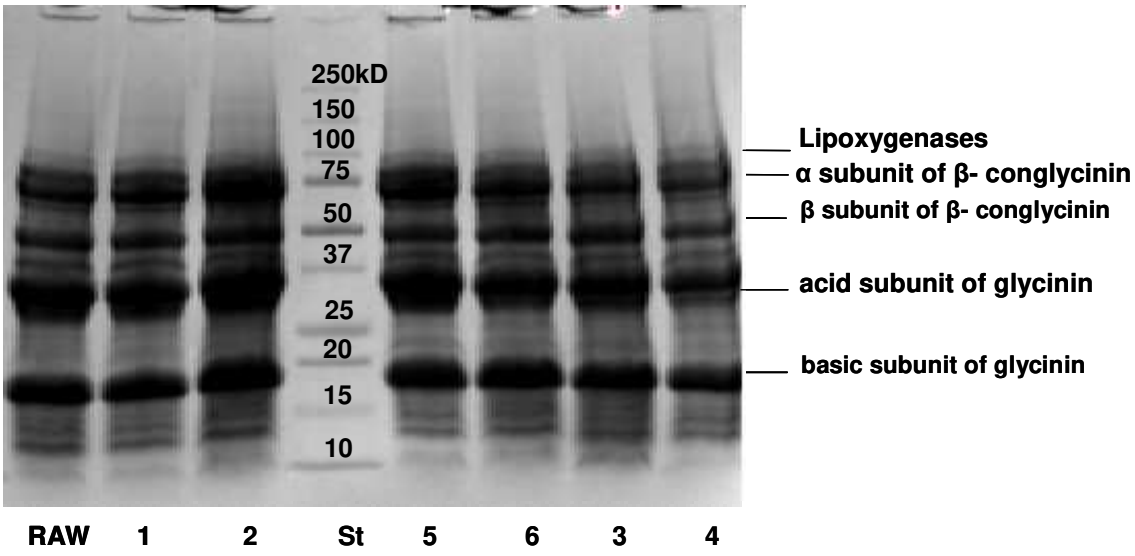


Figure 2. (**A)** Western blot of lunasin for non-germinated soybean (Raw) and experiments: 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 5(12 h, 25 ℃), 6 (72 h, 25 ℃), 3 (21 h, 30 ℃) and 4 (63 h, 30 ℃) (as indicated in Tabl e 3). (**B**) Coommassie Blue staining of protein extraction in a SDS-PAGE electrophoresis gel for non-germinated soybean flour and experiments: 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 6 (72 h, 25 ℃), 3 (21 h, 30 ℃) and 4 (63 h, 30 ℃) (as indicated in **Table 3**).

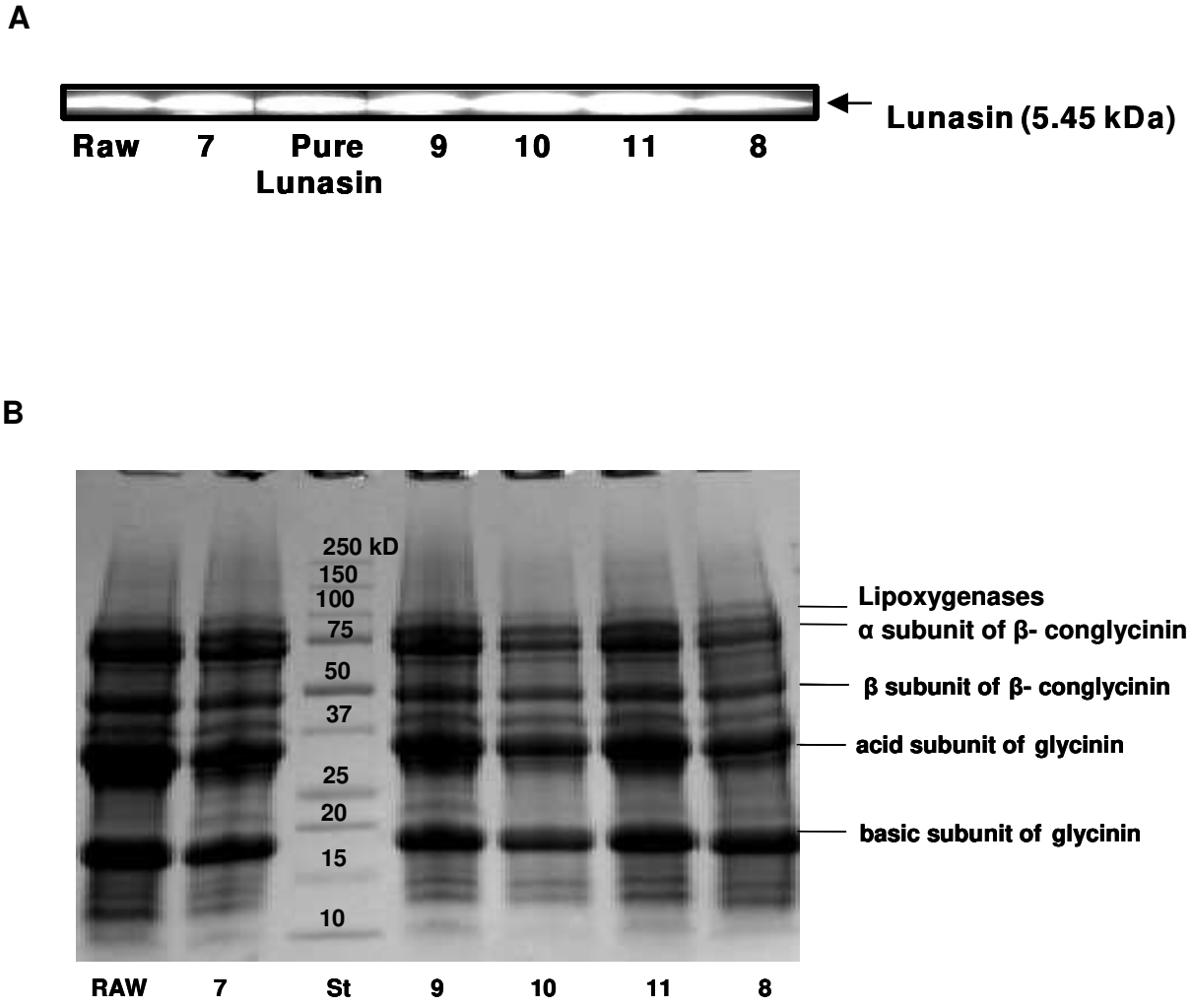


Figure 3. (**A**) Western blot for identification of lunasin for non-germinated soybean (Raw) and experiments: 7 (42 h, 18 ℃), 9 (42 h, 25 ℃), 10 (42 h, 25 ℃), 11 (42 h, 25 ℃) and 8 (42 h, 32 ℃) (as indicated in **Table 3**). (**B**) Coomassie Blue staining of protein extraction in a SDS-PAGE electrophoresis gel for soybean for non-germinated and experiments: 7 (42 h, 18 ℃), 9 (42 h, 25 ℃), 10 (42 h, 25 ℃), 11 (42 h, 25 ℃), and 8 (42 h, 32 ℃) (as indicated in Table 3).

3.6 Isoflavone content

The total isoflavone contentration of the non-germinated freeze-dried soybean flour was 390 mg /100 g of defatted sample, of which 26.69 mg/100g of deffated sample was composed of the aglycones daizein, glycitein and genistein and the total isoflavone contentration of germinated soybean varied from 278.77 to 452.24 mg/100g of deffated samples for the different treatments (**Table 6**). The regression coefficient for the equation obtained for the complete model was 0.90. In this case, the non-significant terms were removed, to make the regression equation simple with an R^2 = 0.72. The second-degree adjusted model in terms of coded factors is presented in Equation (7) and the response surface in **Figure 4A**.

Total isoflavone (mg/100g of deffated sample) = $\mathbf{369.44 - 17.60x_1 - 36.84\ x_2}$ (7)

The highest isoflavones concentration was obtained with lower germination time [12 h (-α) to 42 h (0)] and temperature of 18 ℃ (- α) to 25 ℃ (0) of temperature.

The total aglycone content in germinated soybean flour varied from 8.71 to 90.31 mg /100g of deffated sample for the different treatments. The regression coefficient for the complete model was 0.86. In this case, the non-significant terms were removed to make the regression equation simple with R^2 = 0.85. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Equation (8) and the response surface in **Figure 4B**.

Total isoflavone aglycones (mg/100g of deffated sample) = $\mathbf{26.51 + 12.69x_1 + 15.12x^2 + 19.10\ x_1x_2}$ (8)

Higher concentrations of total aglycone forms were found in germinated soy flours obtained from higher germination time [63 h (+1) to 72 h (+α)] and higher germination temperatures [30 ^{o}C (+1) to 32 ^{o}C (+α)].

Table 6. Isoflavone concentrations in soybean BRS 133 with different times and temperatures of germination.

ISOFLAVONE[1] (mg /100 g)	Raw	1	2	3	4	5	6	7	8	9	10	11
Aglycones												
Daidzein	10.98	5.65	3.53	8.3	45.34	3.12	13.76	2.89	15.2	12.74	12.56	12.98
Genistein	14.40	7.45	5.28	9.9	44.97	3.55	16.72	5.82	17.82	14.53	14.98	14.53
Glycitein	1.31	0	0	0	0	0	0	0	0	0	0	0
β-glucosides												
Daidzin	42.46	65.40	29.48	37.37	57.62	30.86	33.10	36.47	24.09	38.8	40.28	38.57
Genistin	36.12	31.87	32.81	31.16	29.32	27.74	36.87	33.32	23.83	33.9	34.48	35.12
Glycitin	10.40	0	0	0	0	0	0	0	0	0	0	0
Acetylglucosides												
Acetyldaidzin	0	0	0	0	0	0	0	0	0	0	0	0
Acetylgenistin	0	0	0	0	0	0	0	0	0	0	0	0
Acetylglycitin	0	0	0	0	0	0	0	0	0	0	0	0
Malonylglucosides												
Malonyldaidzin	131.62	171.87	139.65	124.25	123.56	165.25	119.38	136.81	115.75	122.94	122.54	122.10
Malonylgenistin	100.75	57.55	36.39	38.16	8.02	57.43	38.06	38.7	35.84	29.59	31.45	29.49
Malonylglycitin	41.96	113.15	148.02	111.21	28.62	116.61	104.15	126.93	46.24	110.66	108.63	110.79
Total aglycone	26.69	13.10	8.81	18.20	90.31	6.67	30.48	8.71	33.02	27.27	27.54	27.51
Total isoflavone	390.00	452.94	395.16	360.35	337.45	404.56	362.04	380.94	278.77	363.16	364.92	363.58

Time and temperature of experiments: 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 3 (21 h, 30 ℃), 4 (63 h, 30 ℃), 5(12 h, 25 ℃), 6 (72 h, 25 ℃), 7 (42 h, 18 ℃), 8 (42 h, 32 ℃), 9 (42 h, 25 ℃), 10 (42 h, 25 ℃), 11 (42 h, 25 ℃).
[1]mg isoflavone/100g deffated soybean flour.

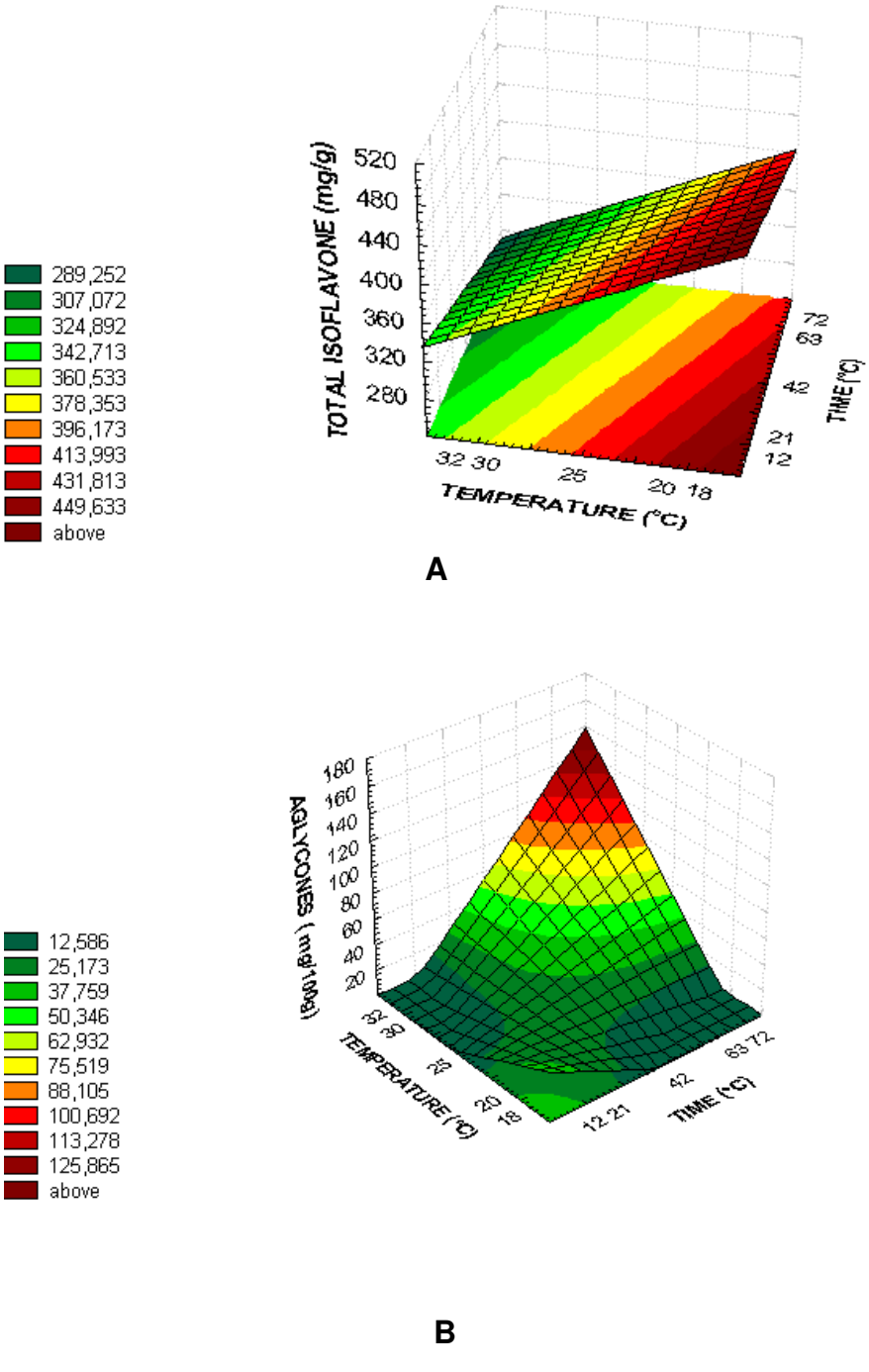


Figure 4. Response surfaces of germination time versus germination temperature for soybean seeds BRS 133. (**A**) Total isoflavones. (**B**) Total aglycones.

The optimal conditions were 63 h of germination time at 30 ℃ resulting in an increase of up to 90.31 mg/100g (238.36%) in these bioactive compounds. In this case, the hydrolysis of glucoside during soaking and germination process contributed to increase genistein levels from 14.40 mg/100g of non-germinated soybean flour to 44.97 mg/100g in germinated soybean flour. When germination time increased to 72 h at 25℃, a decreased in genistein content was observed (16.72 mg/100g) may have been due to the conversion of genistein to other isoflavone forms (Zhu, Hettiarachchy, Horax & Chen, 2005). The acetylglucosides, glycitin and glycitein were not detected within the ranges estudied.

3.7 Saponins content

The total saponins glycoside concentration in the non-germinated freeze-dried soybean flour was 9.75 mg/g and the total saponins concentration in the flours from germinated soybean seeds varied from 8.18 to 12.86 mg/g in the different treatments (**Table 7**). Higher saponins content in germinated soybean seeds has been reported (Bau, Villaume & Méjean, 2000; Zhu, Hettiarachchy, Horax & Chen, 2005). The regression coefficient for the complete model was 0.95. In this case, the non-significant terms were removed to make the regression equation simple with an R^2 = 0.93. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Equation (9) and the response surface in **Figure 5**.

$$\text{Total saponins (mg/g)} = \mathbf{10.83 - 1.58x_1 + 0.46x_2} \qquad (9)$$

Higher values of total saponins were observed at high germination time [63 h (+1) to 72 h (+α)]. The optimal condition was with 63 h germination time at 30℃. In this case, the germination process contributed to an increase in saponins content from 9.75 mg/g in the non-germinated soybean flour to 12.89 mg/g in germinated soybean flour, resulting in a increase of 31.89%.

Table 7. Saponin content in soybean BRS 133 with different times and temperatures of germination.

SAPONINS (mg/g)	Raw	1	2	3	4	5	6	7	8	9	10	11
DDMP & Group B saponins												
Soyasaponins I	1.67	1.55	1.68	1.66	2.08	1.57	1.69	1.68	2.05	2.26	2.31	2.24
Soyasaponins II	0.23	0.13	0.23	0.13	0.14	0.16	0.35	0.24	0.18	0.24	0.24	0.24
Soyasaponins III	0.74	0.67	0.78	0.73	0.96	0.68	0.79	0.77	0.87	0.97	0.96	0.96
Soyasaponins IV	0.13	0.04	0.06	0.04	0.12	0.07	0.17	0.18	0.18	0.18	0.18	0.18
Soyasaponins V	0.29	0.28	0.31	0.29	0.34	0.28	0.29	0.27	0.34	0.35	0.33	0.34
Soyasaponins βg	2.25	1.04	1.65	1.60	6.44	2.05	2.44	2.15	5.38	3.58	3.60	3.64
Soyasaponins βa	0.24	0.21	0.26	0.23	0.23	0.20	0.21	0.12	0.12	0.26	0.27	0.29
Soyasaponins γg	0.94	0.05	0.10	0.11	0.06	0.05	0.15	0.10	0.12	0.08	0.07	0.09
Soyasaponins γa	0.14	2.47	2.26	2.43	0.93	2.03	2.28	2.61	1.04	1.32	1.38	1.30
Soyasaponins αg	0.55	0.17	2.58	0.17	0.32	0.20	2.14	0.50	0.53	0.24	0.24	0.24
Total group B	**7.18**	**6.61**	**9.91**	**7.39**	**11.62**	**7.29**	**10.51**	**8.62**	**10.81**	**9.48**	**9.58**	**9.52**
A group												
Soyasaponins aA1	2.30	1.05	0.96	1.04	0.30	1.01	1.11	1.09	0.34	0.87	0.79	0.84
Soyasaponins aA2	0.23	0.17	0.15	0.29	0.55	0.20	0.41	0.29	0.31	0.17	0.18	0.18
Soyasaponins aA7	0.04	0.35	0.65	0.38	0.39	0.41	0.72	0.66	0.31	0.54	0.55	0.56
Total group A	**2.57**	**1.57**	**1.76**	**1.71**	**1.24**	**1.62**	**2.24**	**2.04**	**0.96**	**1.58**	**1.52**	**1.58**
Total Soyasaponins	**9.75**	**8.18**	**11.67**	**9.10**	**12.86**	**8.91**	**12.75**	**10.66**	**11.77**	**11.06**	**11.10**	**11.10**

Time and temperature of experiments: 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 3 (21 h, 30 ℃), 4 (63 h, 30 ℃), 5(12 h, 25 ℃), 6 (72 h, 25 ℃), 7 (42 h, 18 ℃), 8 (42 h, 32 ℃), 9 (42 h, 25 ℃), 10 (42 h, 25 ℃), 11 (42 h, 25 ℃).

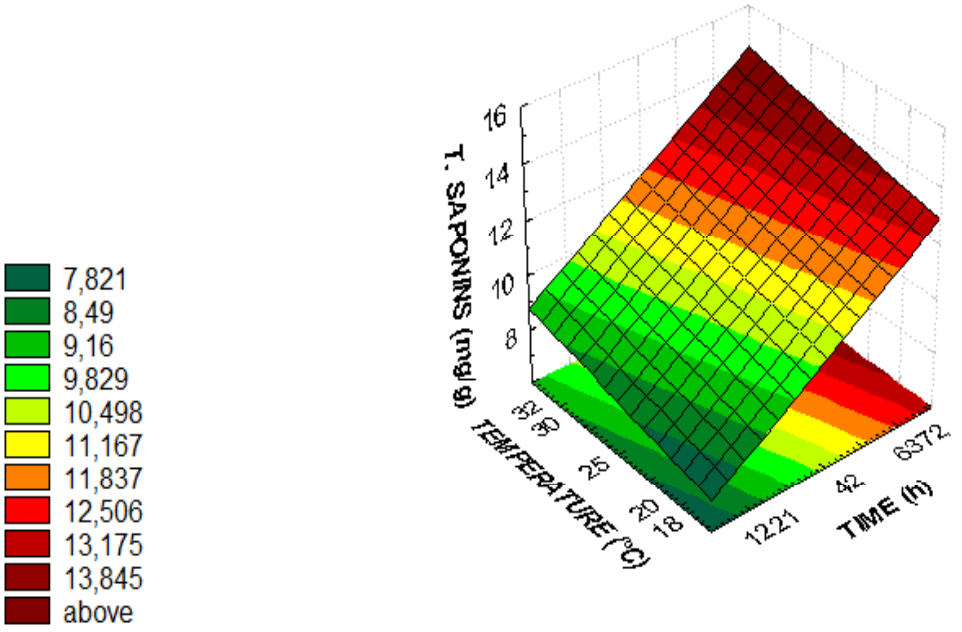


Figure 5. Response surfaces of germination time versus germination temperature for soybean seeds BRS 133, for total saponins.

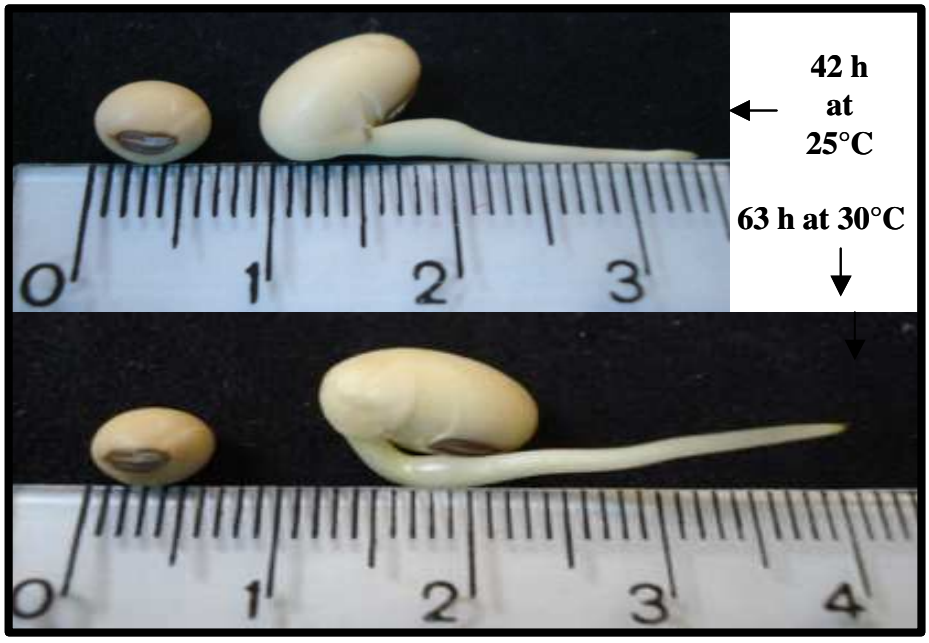


Figure 6. Development of radicules and cotyledons of soybean germinated in the best treatments: 42 h at 25 ℃ and 63 h at 30 ℃.

3.8 Radicles and cotyledons of soybean germinated

The development of radicles and cotyledons of the germinated Brazilian soybean cultivar BRS 133 under the best treatments are as followed: 42 h at 25 ℃ (highest concentration of lunasin and lowest concentration of lectin and lipoxigenase) and 63 h at 30 ℃ (highest concentrat ion of isoflavone aglycones and total saponins) is presented in **Figure 6.**

4. Conclusions

It can be concluded that germination time and temperature had a significant influence on the composition and concentration of bioactive compounds in the germinated soybean flour from the Brazilian soybean cultivar BRS 133, whithin the ranges studied.

The optimal germination conditions for soybean cultivar BRS 133 with high lunasin concentration, low lectin concentration and low lipoxygenase concentration, was exactly the central point, at 25 ℃ during 42 h (0,0).

Germination of soybean cultivar BRS 133 for 42 h at 25 ℃ (0,0) compared with non-germinated soybean, resulted in a significant increase in lunasin concentration from 12.29 to 21.20 mg/g (73.62%), a significant decrease in lectin concentration from 16.96 to 7.62 mg/g (55.07%) and a significant decrease in lipoxygenase activity from 13.3 to 4.0 % (69.92%).

A significant increase in the concentration of isoflavone aglycones (daidzein and genistein) from 26.69 to 90.31 mg/g (238.36%) and total saponins from 9.75 to 12.86 mg/g (31.89%) was observed for 63 h of germination time at 30℃. In relation to genistein concentration in the non-germinated soybean, germination conditions (63 h at 30 ℃) contributed to an increa se from 14.40 to 44.97 mg/100g of deffated sample (212.29%).

5. Acknowledgments

The authors wish to thank CAPES-PEC PG for granting Luz Maria Paucar-Menacho´s scholarship and Mr. Rodolfo Rohr Neto (SoSoja do Brasil Ltda.) and Mr. Kenji S. Narumiya (Sun Foods-Brasil) for the financial support to stay in the Laboratory of Food Science and Human Nutrition of the University of Illinois at Urbana-Champaign. Embrapa-Soybean – The National Center for Soybean Research, Brazil. Embrapa Technology Transfer, Brazil, for the donation of soybean BRS 133. Unicamp's Foundation for Teaching, Research and Extension (FAEPEX) for the grant.

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Capítulo 4

Effect of time and temperature of germination of Brazilian soybean cultivar BRS 258 on the concentration of its bioactive compounds

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This paper was submitted to Journal of Agricultural and Food Chemistry on Nov 29th, 2008

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Abstract

The consumption of soybeans and soybean products has increased in the last decade due to the functional properties of bioactive compounds such as lunasin, Bowman Birk Inhibitor (BBI), lectin, saponins and isoflavones. The objective of this study was to determine the effect of germination of soybean seeds cultivar BRS 258 on its bioactive compounds. Germination was carried out in a germination chamber with paper, samples were frozen at –30 ℃, freeze-dried and milled to produce germinated soybean flour. Isoflavones and saponins were determined by high performance liquid chromatography. Lunasin, BBI and lectin were analyzed by ELISA and Western blot. The effects of the variations in germination time and temperature were analyzed using the Response Surface Methodology (RSM), with a 2^2 central composite rotational design. The independent variables studied were germination time (12, 21, 42, 63 and 72 h) and germination temperature (18, 20, 25, 30, 32℃). The germination conditions of soybe an BRS 258 modified the concentrations of bioactive compounds within the ranges studied and it increased the concentration of lunasin, isoflavone aglycones, saponin glycosides and decreased the concentration of BBI, lectin and lipoxygenase. Optimal increases in the concentrations of the isoflavone aglycones (daidzein and genistein) and the saponin glycosides were observed with a 63 h germination time at 30 ℃. Both germination time and temperature had an influence significant on the composition and concentration the bioactive compounds in germinated soybean flour.

KEYWORDS: Soybean BRS 258; germination; lunasin; Bowman-Birk inhibitor; lectin; isoflavones; saponins.

1. Introduction

Soybean (*Glycine max* (L.) Merril) is consumed by Asian populations and is today advocated for Western diets because of its nutritional benefits (*1*). The use of soybean in human foods has been limited by the presence of several anti-nutritional factors. The majority of processed soybean products have been derived from dry mature soybeans. However, the development of products from germinated soybean presents another option to further increase the versatility and utilization of soybeans. Germination has been identified as an inexpensive and effective technology for improving the nutritional quality of soybean (*2*). Nevertheless, the effects of germination conditions (temperature, light, moisture, and germination time) on bioactive compounds can vary greatly with the plant species, seed varieties or cultivars (*3, 4, 5, 6*).

Soybean is a complex matrix containing several bioactive compounds, including lunasin, Bowman Birk Inhibitor (BBI), isoflavones, saponins, and some other soy proteins and bioactive peptides. Lunasin is a novel cancer preventive 43 amino acid peptide originally isolated from soy (*7, 8*). BBI is a 71 amino acid peptide with 7 disulfide bonds and a double head with the chymotrypsin inhibitor domain located on one of the heads (*9*). Lectin has both antinutritional as well as anti-carcinogenic properties (*10, 11*). Lectin accumulates in seed protein storage vacuoles of cotyledons and is degraded during seed germination and maturation (*12, 13*). The soy lipids are the major source of flavor compounds in soybean protein products. Soybean seeds are a relatively rich source of lipoxygenases, which are an important factor in the generation of odor and off-flavor compounds from lipids and also deteriorate palatability. Short periods of germination (72 h) can substantially improve odor and flavor scores of full fat soybean flour because lipoxygenases activity is reduced during the germination process; hence non-defatted flour of germinated seed would have a more stable shelf-life (*14*).

The major soy isoflavone *β*-glucosides in soybean are genistin and daidzin, glycitin and their malonyl and acetyl conjugates at the C-6 position of the glucose group (*15*). Soybean products may also contain small to large amounts of the aglycone

forms genistein, daidzein and glycitein. Mature soybeans also contain the group A and group B soyasaponins. The group B soyasaponins appear to exist in the intact plant tissue as conjugates of 2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one (DDMP) at the 22-hydroxyl position *(16).* The DDMP conjugates are relatively labile and are easily degraded, most likely resulting in the formation of the non DDMP group B soyasaponins *(17).*

The objective of this study was to evaluate the effects of the variations in germination time (12, 21, 42, 63 and 72 h) and temperature (18, 20, 25, 30, 32℃) in Braziliam soybean cultivar BRS 258, using the Response Surface Methodology (RSM), with a 2^2 central composite rotational design on the concentration of soluble protein, lunasin, BBI, lectin, saponins and isoflavones.

2. Materials and methods

2.1 Materials

Soybean cultivar BRS 258, with a weight of 227 g per 1000 seeds, was developed as part of the breeding program of Embrapa Soybean, Brazil. This cultivar was selected because of its high level of protein and low level of isoflavones (18). Soybean seeds (9.6% moisture) were cleaned with sodium hypochlorite (100 mg/kg) for 10 min, then rinsed three times with distilled water and kept at room temperature for 8 h. Germination was carried out in a germination chamber using paper in trays containing 500 g of seeds each. Germinated seeds were then frozen at –30 ℃ for 4 h, freeze-dried, a nd milled to produce germinated soybean flour obtained in a refrigerated hammer mill, model 680 from Marconi (Piracicaba, Brazil), and the powders stored at 7℃ , conditioned in air-tight glass.

Immunoaffinity purified lunasin (98%) from soy and rabbit polyclonal antibody against the lunasin epitope –EKHIMEKIQGRGDDDDD were provided by Dr. Ben O. de Lumen, University of California at Berkeley. Purified A and B group soy

saponins were prepared in the Peoria laboratory. The primary polyclonal antibody that is specific for lectin from soybean was provided by Dr. Theodore Hymowitz from the Department of Crop Sciences, University of Illinois at Urbana-Champaign. The lectin anti-serum was obtained by immunizing young male New Zealand white rabbits with a subcutaneous injection of 5 ml emulsion containing 5 mg of pure lectin, 1 mL of distilled water and 1 mL of Freund's complete adjuvant. Six week after the first immunization, rabbits showing response to the antibodies (measured 20 days after the first injection) were injected again with a similar dose and bled two weeks later (19).

2.2 Protein extraction

Fifty mg of soybean flour and 1 mL of extracting buffer (0.05M Tris-HCl buffer, pH 8.2) were placed in an Eppendorf tube. After mixing, the samples were sonicated in an ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT) for 70 min, mixing every 10 min to avoid settling, at 40 ℃ using a recirculation bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, NH). The samples were centrifuged at 20,000 g for 40 min at 8 ℃ in an Ep pendorf Centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY), and the supernatant was decanted to a new Eppendorf tube.

2.3 Determination of soluble protein concentration by DC assay

The protein concentration was determined using the BioRad *DC* Microplate Assay Protocol (Bio-Rad Laboratories, Hercules, CA). Briefly, 5 µL of samples (1:20 dilution) were placed in a 96-well plate and treated with 25 µL of Bio-Rad A (alkaline copper tartrate solution) and 200 µL of Bio-Rad reagent B (dilute Folin reagent) (Bio-Rad Laboratories, Hercules, CA). The plate was gently agitated and incubated for 15 min at room temperature. After incubation, the absorbance was

measured at 630 nm. The protein concentration was calculated using pure bovine serum albumin standard curve (μg/mL) (y = 0.0002x - 0.0021, R^2 = 0.997).

2.4 Enzyme-linked immunosorbent assay (ELISA) for lunasin and BBI

Lunasin concentration of germinated soy flour was analyzed by ELISA (*7*) with the following modifications. Samples of 100 µL of protein extracts (1:5,000 dilution) were placed in a 96-well plate and stored for 14 h. Lunasin mouse monoclonal antibody (1:4,000 dilution) was used as the primary antibody and anti-mouse IgG alkaline phosphatase conjugate (1:7,000) (Sigma Chem, St. Louis, MO) as the secondary antibody. The reaction was stopped adding 25 µL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. A similar procedure was used for BBI analysis. Samples of 100 µL of protein extracts (1:10,000 dilution) were placed in a 96-well plate, except that BBI mouse monoclonal antibody (1:1000 dilution) (Agdia, Inc., Elkhart, IN) was used as the primary antibody and anti-mouse alkaline phosphatase (AP) conjugated IgG (1:2,000) as the secondary antibody. Standard curves were determined using purified lunasin (ng/mL) (y= 0.0054x+ 0.001, R^2 = 0.993) and purified BBI (ng/mL) (y= 0.0108x + 0.0465, R^2 = 0.998).

2.5 Enzyme-linked immunosorbent assay (ELISA) for lectin

Lectin concentration in soy flour from germinated seeds was analyzed by ELISA (*10*) with the following modifications. One hundred microliters (100 µL) of protein extract (1:10,000 dilution) was placed in a 96-well plate. Lectin mouse polyclonal antibody (1:500 dilution) were used as the primary antibody, and anti-rabbit IgG alkaline phosphatase conjugate (1:1,000) as the secondary antibody. The reaction was stopped adding 25 µL of 3 N NaOH at 30 min and the absorbance (405 nm) read at 35 min. Standard curves were determined using purified lectin (ng/mL) (y= 0.0101x + 0.0025, R^2 = 0.998).

2.6 Gel Electrophoresis

To the supernatant of each protein extract (20 µL), 20 µL of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with 5% 2-mercaptoethanol were added in Eppendorf tubes which were then heated at 100˚ C for 3 min. The samples (20 µL) and the standard (5 µL) were loaded in the wells of the gel. The gel was run in a Mini Protean-3 cell (Bio-Rad, Laboratories) using 10-20% gradient Tris-Glycine SDS buffer as the running buffer. A 600 Precision Plus Protein standard (Bio-Rad, Laboratories, Hercules, CA) was included as molecular mass marker (lane Std). The power was set at 400 mA (200 V) constant for 30 min. Gels were fixed with peptide fixing solution for 30 min in methanol/acetic acid/water (10:40:50) and were stained with Bio Safe Coomassie G = 250 (Bio-Rad, Laboratories) overnight and the destained with a 10% solution of acetic acid. Gels were read in a Kodak Image Station 440 CF, where the respective molecular masses and band intensities were recorded. Amino acid sequences of major soy proteins were retrieved from UniProtKB/Swiss-Prot Release 54.1 of 21-Aug-2007. The theoretical molecular weight of each protein was calculated from the amino acid sequence with ProtParam program http://ca.expasy.org/tools/protparam.html). Identification of lipoxygenase band (92.9 kDa) was confirmed by comparing the theoretical molecular weight with the experimental data.

2.7 Western Blot procedures

Identity of lunasin was established by Western blot analysis in the protein extract of germinated soybean flours. Samples were centrifuged (20,000 g) at 8 ℃ to eliminate any precipitate. Unstained gels were soaked in 20 mL of blotting buffer (20% methanol, 80% 1x Tris-glycine SDS) for 15 min. A Western blot sandwich was assembled by the following order: a sponge, filter, gel, polyvinylidene difluoride (PVDF) membrane Inmobilon^{TM}-FL (Millipore Corporation), and another filter and sponge, being careful to avoid formation of bubbles, and then developed for 1 h at 110 V and 4 ℃. After the complete trans fer, membrane was then

saturated by incubation in 5% nonfat dry milk (NFDM) in 0.01% TBST (0.1% Tween 20 in Tris-Buffered saline) buffer for 1 h at 4 ℃, and washed three times for 5 min with fresh changes of 0.01% TBST. The washed gel was incubated with lunasin mouse monoclonal antibody (1:1,000 dilution) prepared in 1% NFDM in TBST buffer for 16 h at 4 ℃. After washing the incubated membrane, the membrane Inmobilon^{TM}-FL (Millipore Corporation) was incubated with anti-mouse IgG alkaline phosphatase conjugate (1:10,000 dilution) prepared in 1% NFDM in TBST buffer for 3 h at room temperature. The membrane was prepared for detection using chemiluminescence reagent (Lumigen TM, GE Healthcare, Buckinghamshire, UK).

2.8 Determination of isoflavone concentration by HPLC

Quantitative analysis of isoflavones was carried out following the procedure used by Berhow (*20*). Approximately 250 mg defatted soybean flour was extracted in test tubes with 3.0 mL dimethyl sulfoxide:methanol (1:4 v/v) placed in sealed containers and heated at 50 ℃ for 18 h. The extracts were centrifuged and the supernatants were filtered using 0.45 micron filters. For isoflavone quantification 20 µL aliquots of the extracts were injected into a Shimadzu (Columbia, MD) HPLC system (LC-10AT VP pumps) equipped with a SPDM10A VP photodiode array detector (CTO-10AS VP) and oven column to maintain temperature at 40 ℃, all operating under a Class VP software. Isoflavone separation was carried out in a C18 reverse-phase column YMC – Pack ODS-AM, 250 mm x 4.6 mm, 5 µm particle size (YMC Co, Ltd.). The initial gradient conditions consisted of 100% H_2O containing 0.025% trifluoroacetic acid (TFA), and 0% acetonitrile, to 45% H_2O and 55% acetonitrile, over 25 min. with a flow rate of 1 mL/min. Isoflavones were detected at 260 nm and quantified by comparison with standard curves of genistin, daidzin and glycitin. The concentrations of the malonyl-glucosides and the aglycones were calculated from standard curves of their corresponding *β*-glucosides, using the similarity of the molar extinction coefficients of malonyl-

isoflavones and *β*-glucosides. Isoflavone concentrations were expressed in mg/100 g of defatted samples.

2.9 Determination of saponin concentration by HPLC

Saponin from the soybean flour and germination soybean flour were extracted with dimethylsulfoxide/methanol (1/1) solution at room temperature for 4 h, followed by a 15 min sonication at 50 ˚C and another 2 h extraction at room temperature. The extracts were then filtered through a 0.45 *μ*m nylon filter. HPLC analysis were carried out on a Hewlett-Packard Series 1100 HPLC system equipped with an Inertsil ODS-3 reverse phase C-18 column (250 mm x 4.6 mm ID) and 5 *μ*m particle size, with a metaguard column (Varian) and a G1316A column oven. The system was controlled by HPChem Station version A.06.01. For saponin analysis, a linear water-acetonitrile gradient from 30% to 50% in 45 min was used, with 0.025% TFA added to both solvents. The flow rate was 1 mL/min and the effluent was monitored at 210 nm. Saponins concentrations were calculated by using standard curves prepared from a standardized mix of B group saponins prepared in the Peoria laboratory. The nanomolar extinction coefficient for soyasaponin I, was used to quantitate the remaining B group saponins, the A group saponins, and the DMPP conjugated B group saponins. Identification of saponin peaks was confirmed by comparison to standards and/or LC-MS analysis (*21*).

2.10 Experimental design

Variation effects in germination time and temperature were analyzed using the Response Surface Methodology (RSM), with a 2^2 central composite rotational design. The independent variables studied were: germination time (12, 21, 42, 63 and 72 h) and germination temperature (18, 20, 25, 30 and 32 ºC). Real and coded factor levels for these variables are given in Table 1.

2.11 Statistical analysis

Statistica 5.0 (Statsoft, USA) was used to determine the effects of the independent variables, calculate regression coefficients, carry out analysis of variance (ANOVA) and build the response surfaces, at a 5% significance level.
The following second order polynomial model was fitted to the data:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad (1)$$

Where **Y** is the response variable, X_1 and X_2 are the coded process variables and **βn** are the regression coefficients. A stepwise methodology was followed to determine the significant terms in Eq. 1.

Table 1. Real and coded levels for the independent variables used in the experiments with BRS 258 soybean seeds

Independent variables		Levels				
Coded	Real	-α	-1	0	+1	+α
X_1	Germination time (h)	12	21	42	63	72
X_2	Germination temperature (℃)	18	20	25	30	32

|α| = ±1.41.

3. Results and discussion

Lunasin, BBI and lectin concentrations in non-germinated freeze-dried soybean are presented in **Table 2**. The experimental responses in terms of soluble protein (SP) (mg/g flour), of lunasin (mg/g SP), BBI (mg/g SP), lectin (mg/g SP) and lipoxygenase (%) are presented in **Table 3**. The observed values of soluble protein, lunasin, BBI and lectin in soy flour with different combinations of germination time and temperature are summarized in **Table 4**.

Table 2. Lunasin, BBI and lectin concentrations in non-germinated freeze-dried BRS 258 soybean flour

Components	(mg/g SP)[1]	(mg/g Flour)
Lunasin	14.78 ± 0.13	3.61 ± 0.28
BBI	28.11 ± 0.74	6.86 ± 0.42
Lectin	23.28 ± 0.14	5.68 ± 0.03
Soluble protein	-	244.2

[1] SP= Soluble protein

Table 3. Observed response values with different combinations of germination time and temperature for BRS 258

	Coded level		Response values				
Exp.	X_1 (h)	X_2 (°C)	Soluble Protein (SP) (mg/g flour)	Lunasin (mg/g SP)	BBI (mg/g SP)	Lectin (mg/g SP)	Lipoxy-genase (%)
1	- 1 (21)	-1 (20)	280.4	15.1	25.0	20.3	8.0
2	+1(63)	-1 (20)	330.3	4.6	19.4	9.0	5.4
3	- 1 (21)	+1(30)	311.2	12.2	21.5	18.3	6.2
4	+1 (63)	+1(30)	334.3	5.5	17.0	10.7	2.3
5	-α (12)	0 (25)	272.9	16.4	23.2	21.9	8.9
6	+α (72)	0 (25)	360.0	8.5	17.0	6.0	4.5
7	0 (42)	-α (18)	281.8	4.9	20.4	22.7	6.8
8	0 (42)	+α (32)	332.9	3.3	18.1	20.8	3.5
9	0 (42)	0 (25)	287.6	10.2	17.5	20.5	3.4
10	0 (42)	0 (25)	287.5	11.0	17.4	20.4	3.4
11	0 (42)	0 (25)	288.4	10.8	17.4	20.3	3.4

X_1= Germination time
X_2= Germination temperature
SP= Soluble protein
BBI= Bowman Birk inhibitor

Table 4. Observed values of lunasin, BBI and lectin in soy flour with different combinations of germination time and temperature for BRS 258[1]

Exp.	X_1 (h)	X_2 (℃)	Lunasin (mg/g flour)	BBI (mg/g flour)	Lectin (mg/g flour)
1	- 1 (21)	- 1 (20)	4.2a	7.0a	5.7c
2	+1(63)	- 1 (20)	1.5de	6.4bc	3.0e
3	- 1 (21)	+ 1(30)	3.8b	6.7ab	5.7c
4	+1 (63)	+ 1(30)	1.8d	5.7d	3.6d
5	-α (12)	0 (25)	4.5a	6.3bc	6.0c
6	+α (72)	0 (25)	3.1c	6.1cd	2.2f
7	0 (42)	-α (18)	1.4ef	5.7d	6.4b
8	0 (42)	+α (32)	1.1f	4.8e	6.9a
9	0 (42)	0 (25)	2.9c	5.0e	5.9c
10	0 (42)	0 (25)	3.2c	5.0e	5.9c
11	0 (42)	0 (25)	3.1c	5.0e	5.9c

[1] Means with different superscript letters in the same column are significantly different ($p< 0.05$).
BBI= Bowman Birk inhibitor

3.1 Soluble protein concentration in germinated soy flour

The soluble protein (SP) concentration in the protein extracts from the flours obtained from germinated soybean seeds varied from 272.9 mg/g to 360.0 mg/g flour. The regression model for this parameter was statistically significant ($p < 0.05$, $R^2 = 0.94$). In this case, the non-significant term can be removed to make the regression equation simple with an $R^2= 0.84$. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Equation (2) and the response surface is in **Figure 1A**.

Soluble Protein (mg/g flour)=$\mathbf{297.52 + 24.52x_1 + 11.81x_1^2 + 13.38x_2}$ (2)

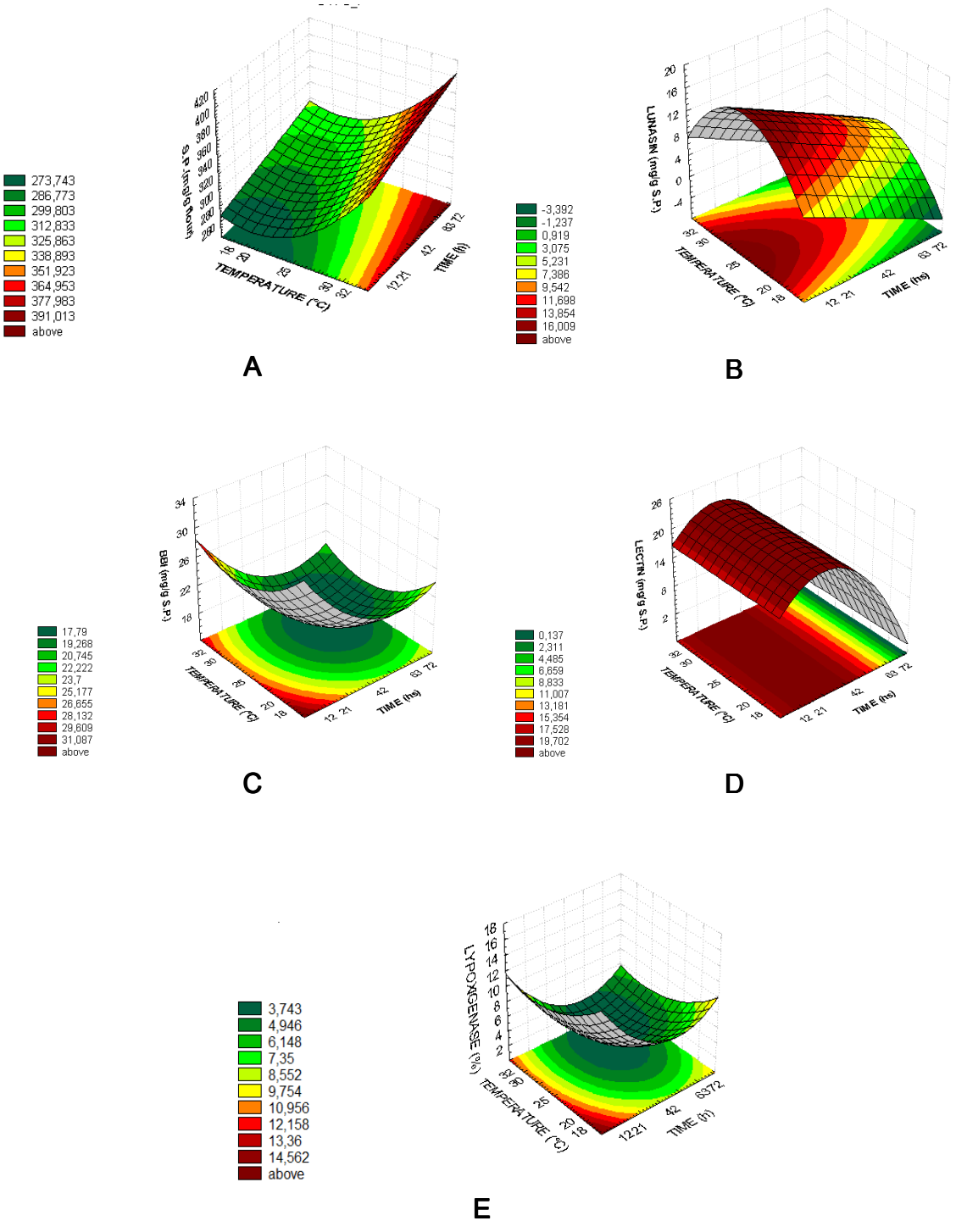


Figure 1. Response surfaces soybean germinated seed BRS 258 flour showing time versus temperature. (**A**) Soluble protein. (**B**) Lunasin. (**C**) BBI. (**D**) Lectin. (**E**) Lipoxygenase.

High values of SP were observed with high germination times of 72 h (+*α*) and germination temperatures from and 18 ℃ (-*α*) to 32 ^{o}C (+*α*) of germination temperature. An increase of temperature from 18 ℃ (-1) to 32 ℃ (+1) at 42 h (0) (comparing Exp 7 and 8) increased the concentration of SP by 18.4% **(Table 3)**. Maintaining the germination temperature at 25 ℃ (0) (Comparing Exp. 5 and 6) an increase in germination time from 12 h (-α) to 72 h (+α), promoted an increase in the concentration of SP by 31.9% in the germinated soybean flour.

3.2 Lunasin Identity and Lunasin Concentration in Extracted Protein

Identification of the lunasin band (5.45 kDa) was confirmed by western blot analysis. The results for lunasin were similar to those reported for different soybean genotypes by Gonzales de Mejia et al. (*7*) (**Figures 2A** and **3A**). The lunasin concentration in the protein extracts from the flours obtained from germinated soybean varied from 3.3 to 16.4 mg/g SP. The regression coefficient for the complete model was 0.96. In this case, the non-significant term can be removed to make the regression equation simple with an R^2 = 0.89. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Equation (3) and the response surface in **Figure 1B**.

$$\text{Lunasin (mg/g SP)} = \mathbf{11.76 - 3.55x_1 - 3.36x_2^2} \qquad (3)$$

Higher values of lunasin were observed at 12 h (-*α*) than 42 h (0) of germination time, and also at 20 ^{o}C (-1) in comparison to 30 ^{o}C (+1) of germination temperature. **Table 3** also shows that a low germination time of 12 h (-α) at 25 ℃ promoted the highest lunasin concentration. This is in agreement with results obtained in the field at 23 ^{o}C in comparison to higher or lower temperatures (*22*).

3.3 Bowman Birk inhibitor concentration in extracted protein

The BBI concentration of the non-germinated freeze-dried soybean flour was 28.1 mg/g SP. The process of germination decreased the BBI concentration in the

protein extracts from the germinated flour, which varied from 17.0 to 25.0 mg/g SP. The regression coefficient for the complete model was 0.96. In this case, the non-significant interaction term can be removed to make the regression equation a 2nd order adjusted model for BBI concentration with an R^2 = 0.95. This is presented in Equation (4) and the response surface in **Figure 1C**.

Bowman Birk inhibitor(mg/g SP) = $\mathbf{17.46 - 2.35x_1 + 1.57x_1^2 - 1.13\ x_2 + 1.15\ x_2^2}$(4)

Lower values of BBI in SP were observed at higher germination temperatures [25 ^oC (0) to 32 ^oC (+α)]. During germination the concentration of protease inhibitors in general (BBI in particular) decreases as a result of BBI digestion by proteases K1 and B2 (*23*). During the course of soybean germination, protease K1 initiates the degradation of BBI followed by extensive proteolysis by protease B2 (*24*). At 63 h of germination (+1) (comparing Exp 2 and 4) (**Table 3**), an increase of temperature from 20 ℃ (-1) to 30 ℃ (+1), promoted a decrease of 12.4% in BBI. Maintaining the germination temperature at a constant 25 ℃ (0) (Comparing Exp. 5 and 6) an increase in germination time from 12 h (-α) to 72 h (+α) promoted decrease of 27.0% in BBI. Seed germination reduces BBI concentration.

3.4 Lectin concentration in extracted protein

The lectin concentration in the non-germinated freeze-dried soybean flour was 23.3 mg/g SP. Germination resulted in decreased lectin concentration in the protein extracts of germinated flours, which varied from 6.0 to 22.7 mg/g SP. The regression coefficient for the complete model was 0.94; but in this case, the non-significant terms can be removed to make the regression equation simple with an R^2 = 0.92. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Equation (5) and the response surface in **Figure 1D**.

Lectin (mg/g SP) = $\mathbf{20.28 - 5.17\ x_1 - 4.02\ x_2^2}$ (5)

Lower values of lectin concentration in SP were observed as germination time increased. This could be an important factor of germination on improving the biological and nutritional value of germinated soybeans for their utilization in human foods and animal feeds (*25*). After 21 h germination (+1) (comparing Exp 1 and 3 in **Table 3**), an increase of temperature from 20 ℃ (-1) to 30 ℃ (+1) resulted in a decrease of 9.9% in lectin concentration. Similarly, maintaining the germination temperature constant at 25℃ (0) (Comparing Exp. 5 and Exp. 6), an increase in germination time from 12 h (-α) to 72 h (+α) promoted a decrease of 72.6% in lectin concentration.

3.5 Lipoxygenase concentration (%)

The identification of the lipoxygenase band (92.9 kDa) was confirmed by comparing the theoretical molecular weight with the experimental data (**Table 5**) and is shown in **Figures 2B** and **3B**. The lipoxygenase concentration of the germinated soybean flours varied from 2.3 to 8.9%, while the lipoxygenase concentration of the non-germinated freeze-dried soybean flour was 11.3%.

The regression model for this parameter was statistically significant ($p < 0.05$) and had an $R^2 = 0.99$. The 2nd order adjusted model ($R^2 = 0.98$) for lipoxygenase concentration is presented in Equation (6) and the response surface in **Figure 1E**.

$$\text{Lipoxygenase (\%)} = \mathbf{3.42 - 1.59\ x_1 + 1.52\ x_1^2 - 1.18\ x_2 + 0.75\ x_2^2} \qquad (6)$$

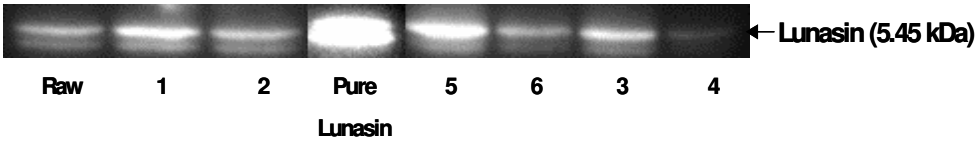
Lower values of lipoxygenase in SP were observed with higher germination temperatures from 25 ^{o}C (0) to 32 ^{o}C (+*α*). Commercial full-fat soy flour has no lipoxygenase activity and the stability of its lipid composition is constant (*14*). Germination induced an increase of protein concentration and caused a reduction in the level of specific activity of lipoxygenase 1 (*26*). After 63 h of germination (+1) (comparing Exp 1 and 3 in **Table 3**), a temperature increase from 20 ℃ (-1) to 30 ℃ (+1) resulted in the decrease of lipoxygenase activity by 22.5%.

Table 5. Calculated molecular masses of major soy proteins[1]

	Name	Accession number	Number of aa	Molecular mass (Da)
Lipoxygenase	1			89100.0
Lipoxygenase	2 and 3			92900.0
	α´ subunit	gi|9967361	554	65142.6
β-conclycinin	α subunit	gi| 9967357	543	63164.8
	β subunit	gi| 9967359	416	47975.7
	G1 precursor	P04776	495	55706.3
	A1 a chain	CHAIN_20-306	287	32646.9
	Bx chain	CHAIN_311-490	180	19955.5
	G2 precursor	P04405	**485**	**54390.7**
	A2 chain	CHAIN_19-296	278	31622.8
	B1a chain	CHAIN_301-480	180	19773.2
Glycinin	G3 precursor	P11828	**481**	**54241.7**
	A chain	CHAIN_22-296	275	31483.7
	B chain	CHAIN_297-476	180	19911.4
	G4 precursor	P02858	**562**	**63587.1**
	A5 chain	CHAIN_24-120	97	10540.8
	A4 chain	CHAIN_121-377	257	29953.9
	B3 chain	CHAIN_378-562	186	20743.5
	G5 precursor	P04347	**516**	**57956.1**
	A3 chain	CHAIN_25-344	320	36392.4
	B4 chain	CHAIN_345-516	172	19049.5

[1] Amino acid sequences of major soy proteins were retrieved from UniProtKB/Swiss-Prot Release 54,1 of 21-Aug-2007, and the theoretical molecular masses of each protein was calculated using the ProtParam program (http://ca.expasy.org/tools/protparam.html).

A



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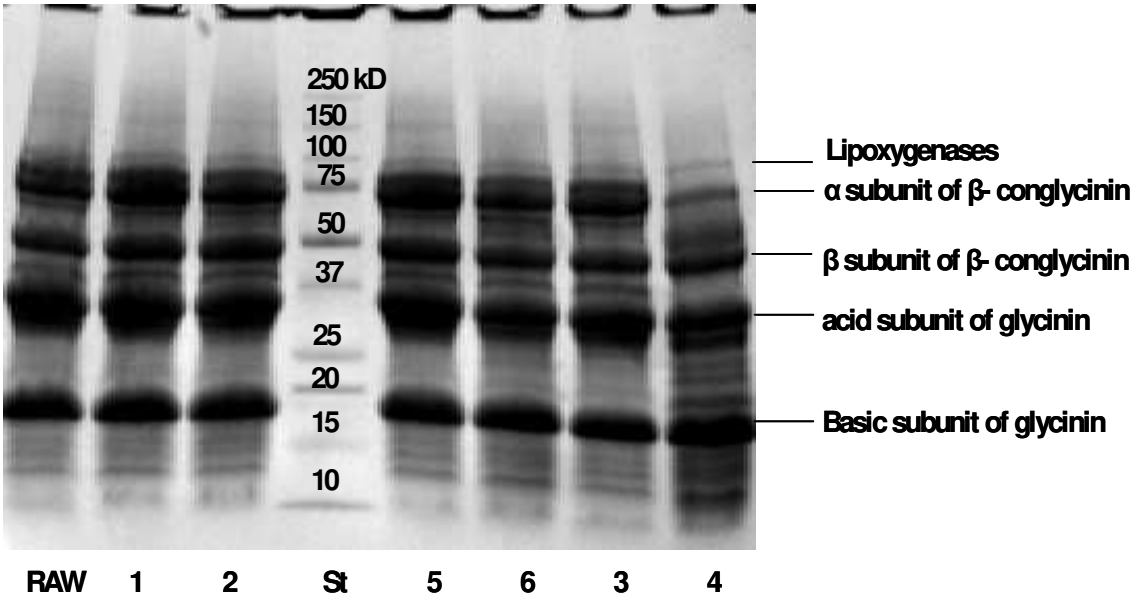


Figure 2. (**A**) Western blot for lunasin in non-germinated soybean and in Experiments 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 5(12 h, 25 ℃), 6 (72 h, 25 ℃), 3 (21 h, 30 ℃) and 4 (63 h, 30 ℃) (as indicated in **Table 3**). (**B**) Coomassie Blue staining of protein extracts in a SDS-PAGE electrophoresis gel for non-germinated soybean flour and for experiments 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 6 (72 h, 25 ℃), 3 (21 h, 30 ℃) and 4 (63 h, 30 ℃) (as indicated i n **Table 3**) of soybean flours prepared from soybeans germinated with different times and temperatures. A Precision Plus Protein standard was included as molecular mass marker (lane Std).

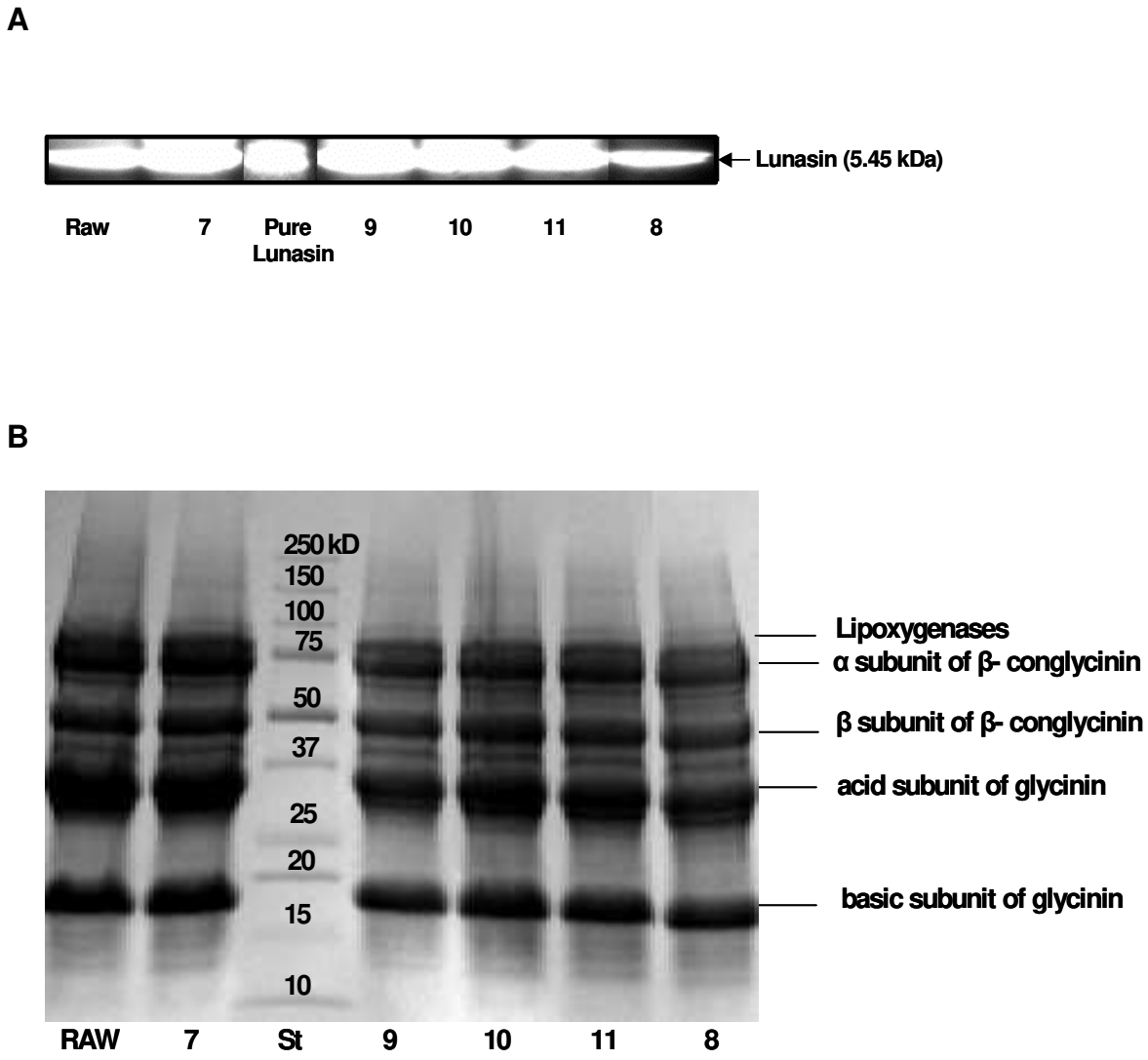


Figure 3. (**A)** Western blot for identification of lunasin in non-germinated soybean and in Experiments 7 (42 h, 18 ℃), 9 (42 h, 25 ℃) , 10 (42 h, 25 ℃), 11 (42 h, 25 ℃) and 8 (42 h, 32 ℃) (as indicated in **Table 3**). (**B)** Coomassie Blue staining of protein extracts in a SDS-PAGE electrophoresis gel for non-germinated soybean or and for Experiments 7 (42 h, 18 ℃), 9 (42 h, 25 ℃), 10 (42 h, 25 ℃), 11 (42 h, 25 ℃), and 8 (42 h, 32 ℃) (as indicated in **Table 3**) of soybean flour germinated with different times and temperatures. A Precision Plus Protein standard was included as molecular mass marker (lane Std).

3.6 Isoflavone concentrations

The total isoflavone concentration of the non-germinated freeze-dried soybean flour was 222.4 mg/100 g of defatted sample, of which 25.4 mg/100 g of defatted sample was composed of the aglycones daizein, glycitein and genistein. The total isoflavone concentration of germinated soybean flours varied from 232.9 to 294.8 mg/100 g of defatted samples for the different treatments, increasing with longer germination times (**Table 6**). The regression coefficient for the equation obtained for the complete model was 0.97. In this case, the non-significant terms were removed, to make the regression equation simple with R^2 = 0.93. The regression second-degree adjusted model in terms of coded factors is presented in Equation (7) and the response surface in **Figure 4A**.

Total isoflavones (mg/100 g of defatted sample) = $\mathbf{241.78 + 11.63x_1^2 - 14.29x_2 + 11.67x_2^2 - 16.05x_1x_2}$ (7)

The highest isoflavone concentrations were achieved with longer germination times from 63 h (+1) to (+α) 72 h, and in the temperature range of 18 °C (- α) to 20 °C (-1).

The total aglycone concentration of germinated soybean flour varied from 4.6 to 64.5 mg/100 g of defatted samples for the different treatments.

The regression coefficient for the complete model was 0.95; in this case, the non-significant terms were removed to make the regression equation simpler, with R^2 = 0.92. The regression equation obtained for the second-degree adjusted model in terms of coded factors is presented in Equation (8) and the response surface in **Figure 4B**.

Total aglycones (mg/100 g of defatted sample)=$\mathbf{22.62 + 6.27x_1 + 12.02x_2 + 15.31\ x_1x_2}$ (8)

Higher concentrations of total isoflavone aglycones were found in germinated soy flours ranging from 63 h (0) to (+α) 72 h of germination time and in the temperature ranges of 30 ℃ (0) to 32 ℃ (+ α). The optimal conditions were 63 h of germination time at 30 ℃, resulting in an increase of up to 64.5 mg/100 g (153.93%) in these bioactive aglycones. In this case, the hydrolysis of the glucosides during the soaking and germination processes contributed to an increase in genistein levels from 15.4 mg/100 g in the non-germinated soybean flour to 47.1 mg/100 g in germinated soybean flour. This result is very good, because the biological properties are predominant when the isoflavones are present as aglycones instead of β-glycosides. When germination time increased to 72 h at 25 ℃, the genistein concentration decreased (21.0 mg/100 g) which may have been due to the conversion of genistein to other isoflavones (*27*). The acetylglucosides forms and glycitein were not detected within the ranges studied.

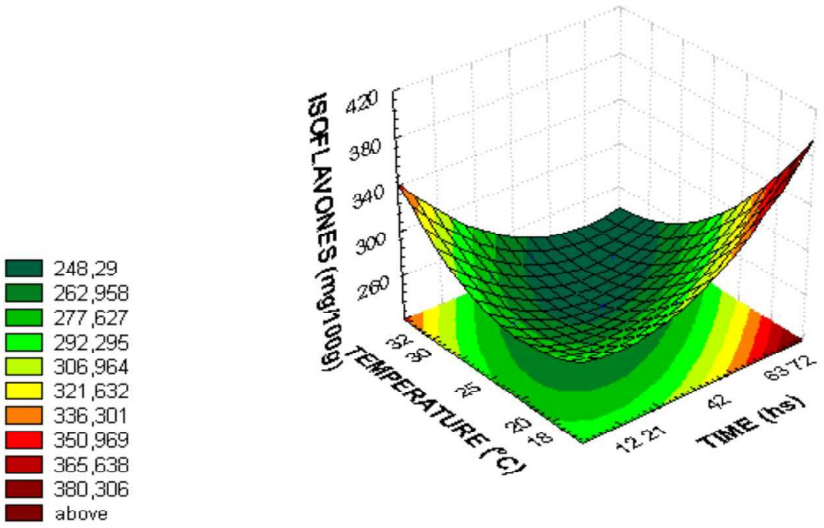
3.7 Saponin concentrations

The total saponin glycoside concentration in the non-germinated freeze-dried soybean flour was 7.4 mg/g. The total saponin concentrations in the flours from germinated soybean seeds varied from 6.7 to 23.5 mg/g in the different treatments (**Table 7**). The regression model for total saponins was not significant (R^2=0.51) within the ranges studied. Higher saponin concentrations in germinated soybean seeds have been reported (25, 28). Yet, the effect of germination on the distribution of the various forms of the soybean saponin glycosides has not been examined. The total saponin concentration increased significantly with the germination time of 63 h at 30℃, to 23.5 mg/g, resulting in a significant increase of up to 215.86% of these bioactive compounds in relation to the non-germinated soybean flour.

Table 6. Isoflavone concentrations in soybean BRS 258 at different germination times and temperatures[1]

ISOFLAVONES (mg/100 g deffated sample)	Raw	1	2	3	4	5	6	7	8	9	10	11
Aglycones												
Daidzein	7.69	5.8	1.3	4.5	17.33	6.4	9.0	1.3	11.4	6.3	5.5	6.2
Genistein	15.41	11.4	3.4	11.3	47.14	13.6	21.0	5.9	22.5	12.6	12.5	12.5
Glycitein	2.30	0	0	0	0	0	0	0	0	0	0	0
β-glucosides												
Daidzin	14.71	15.8	12.7	12.7	9.32	13.9	12.6	11.4	13.3	9.6	9.7	9.7
Genistin	23.09	28.4	25.3	20.6	18.42	22.2	21.0	14.7	20.3	18.4	18.0	17.2
Glycitin	6.02	4.1	3.7	2.8	1.93	4.8	3.7	0.6	0.8	2.8	3.2	3.9
Acetylglucosides												
Acetyldaizin	0	0	0	0	0	0	0	0	0	0	0	0
Acetylgenistin	0	0	0	0	0	0	0	0	0	0	0	0
Acetylglycitin	0	0	0	0	0	0	0	0	0	0	0	0
Malonylglucosides												
Malonyldaidzin	57.56	70.5	95.7	75.3	49.52	70.4	70.7	95.8	63.5	62.2	64.7	65.7
Malonylgenistin	72.96	102.4	114.7	108.6	76.16	96.0	104.0	102.2	89.3	103.9	104.5	104.6
Malonylglycitin	22.64	20.0	38.0	24.8	13.12	32.2	35.2	55.9	28.2	25.2	23.4	23.1
Total aglycone	25.40	17.3	4.6	15.9	**64.5**	20.0	30.0	7.2	33.9	18.9	18.0	18.6
Total isoflavone	222.37	258.4	294.8	260.7	232.9	259.5	277.3	287.9	249.1	241.0	241.6	242.8

[1]Time and temperature of experiments: 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 3 (21 h, 30 ℃), 4 (63 h, 30 ℃), 5 (12 h, 25 ℃), 6 (72 h, 25 ℃), 7 (42 h, 18 ℃), 8 (42 h, 32 ℃), 9 (42 h, 25 ℃), 10 (42 h, 25 ℃), 11 (42 h, 25 ℃).



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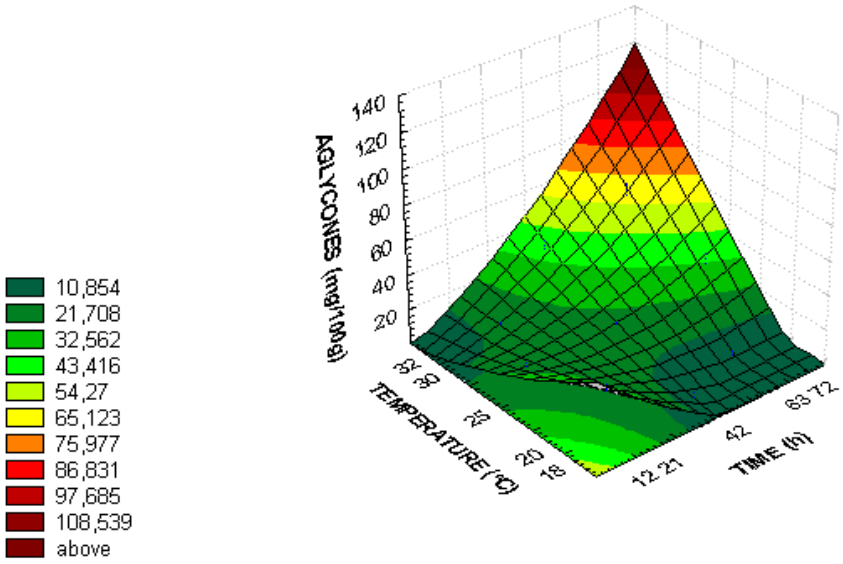

B

Figure 4. Response surfaces of germination time versus germination temperature for soybean seeds BRS 258. (**A**) Total isoflavones. (**B**) Total aglycones.

Table 7. Saponin concentrations in soybean BRS 258 at different germination times and temperatures[1, 2]

SAPONINS (mg/g flour)	Raw	1	2	3	4	5	6	7	8	9	10	11	LSD (p< 0.05)
DDMP & Group B saponins													
Soyasaponin I	1.26	1.4c	1.5bc	1.4c	2.0a	1.4c	1.7b	1.5bc	1.5bc	1.5bc	1.5c	1.5bc	0.19
Soyasaponin II	0.22	0.2c	0.3b	0.2c	0.8a	0.2d	0.2d	0.2d	0.2c	0.2c	0.2c	0.2c	0.02
Soyasaponin III	0.27	0.3c	0.4c	0.3c	1.0a	0.6b	0.6b	0.3c	0.4c	0.4c	0.3c	0.4c	0.06
Soyasaponin IV	0.10	0.1d	0.1d	0.0e	0.9a	0.1bc	0.1b	0.0e	0.1d	0.1d	0.1cd	0.1d	0.02
Soyasaponin V	0.21	0.2c	0.2cd	0.2e	0.3a	0.3ab	0.3b	0.2c	0.2c	0.2c	0.2de	0.2de	0.01
Soyasaponin βg	1.69	1.8bc	1.8bc	1.5de	2.3a	1.5de	1.7bc	1.7bc	1.3e	1.6cd	1.6cd	1.6cd	0.20
Soyasaponin βa	0.12	0.1d	1.1a	0.1bc	0.2bcd	0.2bcd	0.3b	0.2bcd	0.2bc	0.3bc	0.3bc	0.2bc	0.12
Soyasaponin γg	0.57	0.7a	0.6a	0.5bc	0.4dc	0.6ab	0.4dc	0.3ab	0.2e	0.6a	0.6a	0.6a	0.12
Soyasaponin γa	0.09	0.1bc	0.2abc	0.1bc	0.3a	0.6bc	0.3ab	0.2abc	0.1c	0.2abc	0.2abc	0.2abc	0.17
Soyasaponin αg	0.42	0.3c	0.4c	0.3c	0.9a	0.5b	0.5b	0.2e	0.2e	0.2e	0.2e	0.3e	0.15
Total group B	**4.95**	**5.2**	**6.6**	**4.6**	**9.1**	**6.0**	**6.1**	**4.8**	**4.4**	**5.3**	**5.2**	**5.3**	
Group A acetyl-saponins													
Soyasaponin aA_1	2.29	1.8d	3.2b	2.4c	14.0a	0.9e	0.9e	1.6d	2.3c	2.4c	2.4c	2.5c	0.38
Soyasaponin aA_2	0.14	0.2b	0.2cd	0.0f	0.3a	0.1d	0.1d	0.2e	0.1bc	0.0g	0.0g	0.0g	0.02
Soyasaponin aA_7	0.06	0.1abcd	0.1abcd	0.1def	0.1a	0.1ab	0.0f	0.1ef	0.1cdef	0.1cdef	0.1bcde	0.1cedf	0.2
Total group A	**2.49**	**2.1**	**3.5**	**2.5**	**14.4**	**1.1**	**1.0**	**1.9**	**2.5**	**2.5**	**2.5**	**2.6**	
Total Saponins (A+B)	**7.44**	**7.3c**	**10.1b**	**7.1cde**	**23.5a**	**7.1e**	**7.1e**	**6.7de**	**6.9de**	**7.8c**	**7.7c**	**7.9c**	0.76

[1]Means with different superscript letters in the same row are significantly different, $p < 0.05$.

[2]Times and temperatures of experiments: 1 (21 h, 20 ℃), 2 (63 h, 20 ℃), 3 (21 h, 30 ℃), 4 (63 h, 30 ℃), 5(12 h, 25 ℃), 6 (72 h, 25 ℃), 7 (42 h, 18 ℃), 8 (42 h, 32 ℃), 9 (42 h, 25 ℃), 10 (42 h, 25 ℃), 11 (42 h, 25 ℃).

3.8 Radicules and cotyledons of germinated soybean

The development of radicules and cotyledons of the germinated Brazilian soybean BRS 258 with the best treatments are as follows: 42 h at 25 ℃ (lowest concentration of BBI and lipoxygenase), 63 h at 30 ℃ (highest concentration of isoflavones and saponin aglycones) and 72 h at 25 °C (lowest concentration of lectin) (**Figure 5)**.

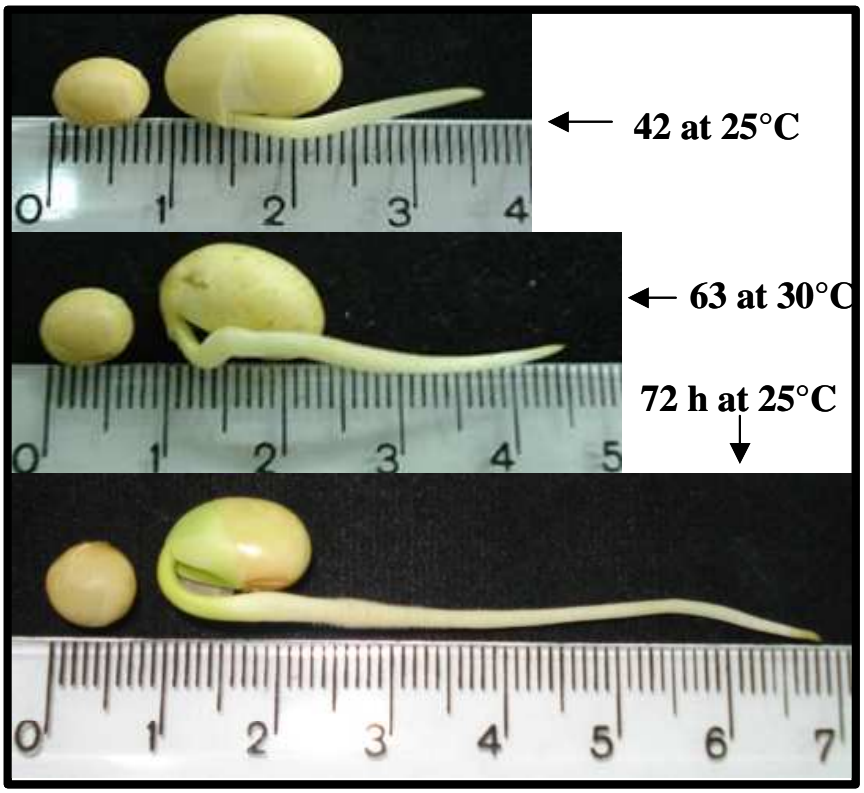

Figure 5. Development of radicules and cotyledons of soybean germinated at best treatments: 42 h at 25 ℃, 63 h at 30 ℃ and 72 h a t 25 ℃.

4. Conclusions

In conclusion, both germination time and temperature had a significant influence on the composition and concentrations of bioactive compounds in germinated soybean flour from the Braziliam soybean cultivar BRS 258 whithin the ranges studied. Germination at 25 ℃ (0) during 12 h (-α) resulted in the highest lunasin concentration. An increase in germination time from 12 h (-α) to 72 h (+α) at 25 ℃ resulted in an increase in soluble protein concentration from 272 to 360 mg/g (31.9%), a decrease in BBI concentration from 23.3 to 17.0 mg/g (27.0%), a decrease in lectin concentration from 21.9 to 6.0 mg/g (72.6%) and a decrease in lipoxygenase activity (%) from 8.9 to 4.5 (49.4%).

Germination of soybean cultivar BRS 258, at 25 ℃ f or 42 h compared with raw soybean flour, resulted in a significant decrease in lipoxygenase activity from 11.3 to 3.4% (69.9%).

A significant increase in the concentration of isoflavone aglycones (daidzein and genistein) from 25.40 to 64.5 mg/g (153.93%) and of total saponins from 7.44 to 23.5 mg/g (215.86 %) was observed in soybean flour germinated at 30 ℃ during 63 h. Compared to genistein concentration on the non-germinated soybean, germination conditions at 30 ℃ for 63 h contribute d to an increase from 15.41 to 47.14 mg/g (205.97%).

5. Acknowledgements

The authors wish to thank CAPES-PEC PG for granting Luz Maria Paucar-Menacho´s scholarship and Mr. Rodolfo Rohr Neto (SoSoja do Brasil Ltda.) and Mr. Kenji S. Narumiya (Sun Foods-Brasil) for the financial support. Embrapa-Soybean – The National Center for Soybean Research, Brazil. Embrapa Technology Transfer, Brazil, for the donation of soybean BRS 258. Unicamp's Foundation for Teaching, Research and Extension (FAEPEX) for the grant.

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Conclusão Geral

Os resultados obtidos neste trabalho permitiram concluir que as variações no tempo e temperatura de germinação tiveram uma influência significativa sobre a composição e as concentrações de compostos bioativos na farinha de soja germinada.

A caracterização físico-química das duas cultivares de soja brasileira permitiu concluir que, embora a sua composição esteja dentro de uma gama típica de nutrientes da soja, surge um padrão distinto de alguns nutrientes e de compostos bioativos no que diz respeito ao teor de proteínas. A cultivar BRS 133 apresentou um baixo teor de proteína e uma alta concentração de isoflavonas totais e, em contrapartida, a cultivar BRS 258 apresentou um alto teor de proteína e baixa concentração de isoflavonas totais.

Na cultivar BRS 133, um tempo de germinação de 42 horas a 25 ℃ resultou em um aumento de 73,62% na concentração de lunasina, uma diminuição de 55,07% na concentração de lectina e uma diminuição de 69,92% na atividade de lipoxigenase. Aumentos significativos nas concentrações de isoflavonas agliconas (daidzeína e genisteína) e saponinas totais foram observados com um tempo de germinação de 63 h a uma temperatura de 30 ℃. Em relação à concentração de genisteína, comparada com o grão de soja sem germinar, a combinação de 63h de germinação a 30 ℃ contribuiu com um aumento de 212,29% neste flavonóide bioativo.

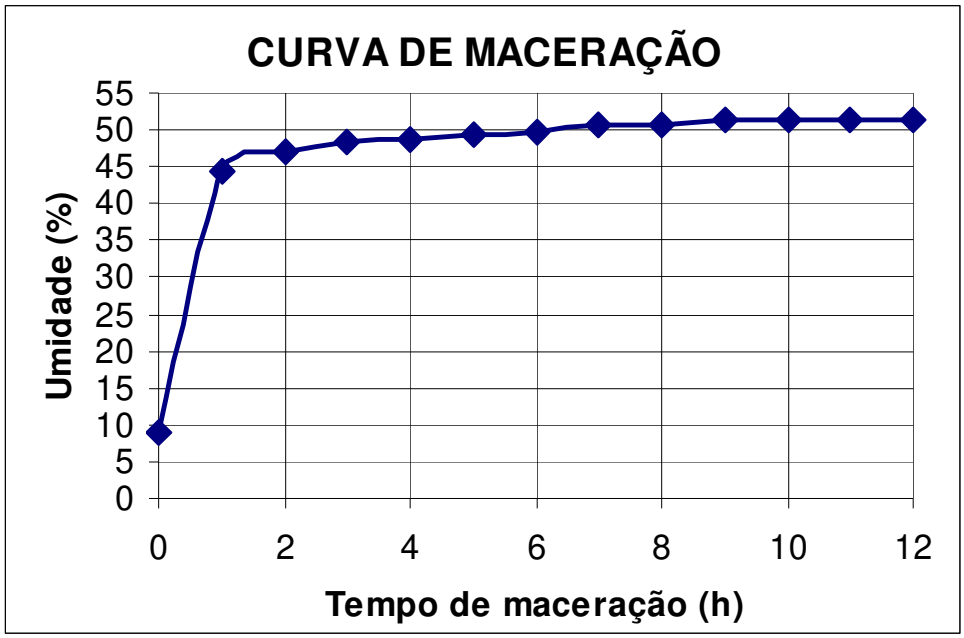
Na cultivar BRS 258, o processo germinativo resultou numa redução de BBI, lectina e atividade de lipoxigenase. Um baixo tempo de germinação de 12 h (-1) a 25 ℃ resultou em maior concentração de lunasina. Um aumento no tempo de germinação de 12 h (-1) a 72 h (+1) a 25 ℃ resultou em um aumento de 31,9% no teor de protéina solúvel, um decréscimo de 27,0% na concentração de BBI, e uma

diminuição de 72,6% na concentração de lectina. Neste cultivar, aumentos significativos nas concentrações de isoflavonas agliconas (daidzeína e genisteína) (153.93%) e de saponinas totais (215,86%) foram observados com um tempo de germinação de 63 h a 30℃. Em relação à concentraçã o de genisteína, comparada com o grão de soja sem germinar, a combinação de 63 h de germinação a 30 ℃ contribuiu com um aumento de 205,97% neste flavonóide bioativo.

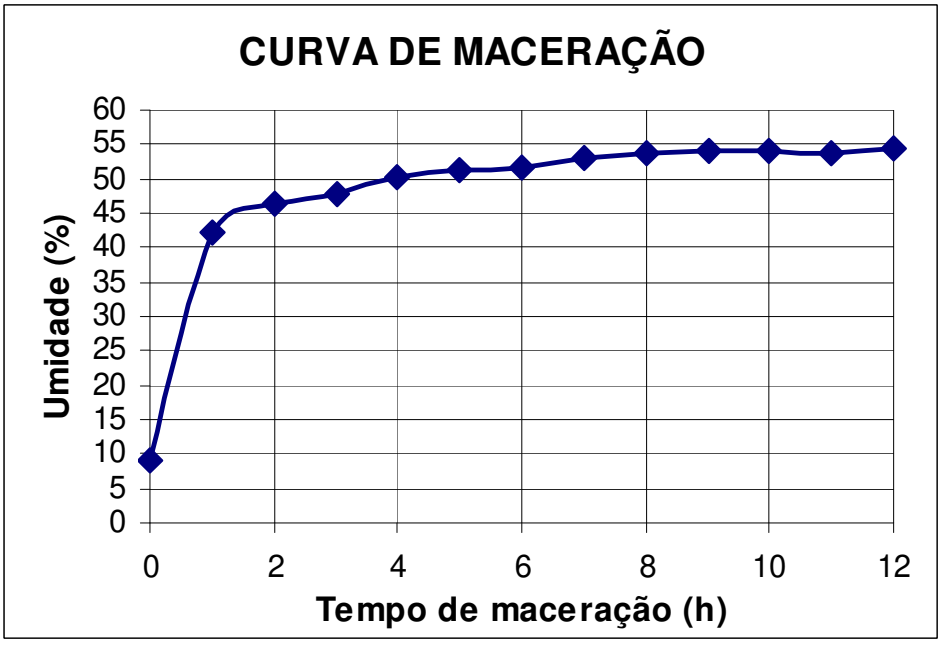
Tanto para a cultivar BRS133 como para a cultivar BRS 258, a combinação de 63 h de germinação a uma temperatura de 30 ℃, poss ibilitam um aumento nas concentrações dos compostos bioativos não protéicos como as isoflavonas e as saponinas.

Baseado neste estudo, condições ótimas do processo de germinação possibilitam a obtenção de farinhas de soja germinadas com propriedades funcionais, o que possibilita sua aplicação em formulações de inúmeros produtos alimentícios com benefícios à saúde.

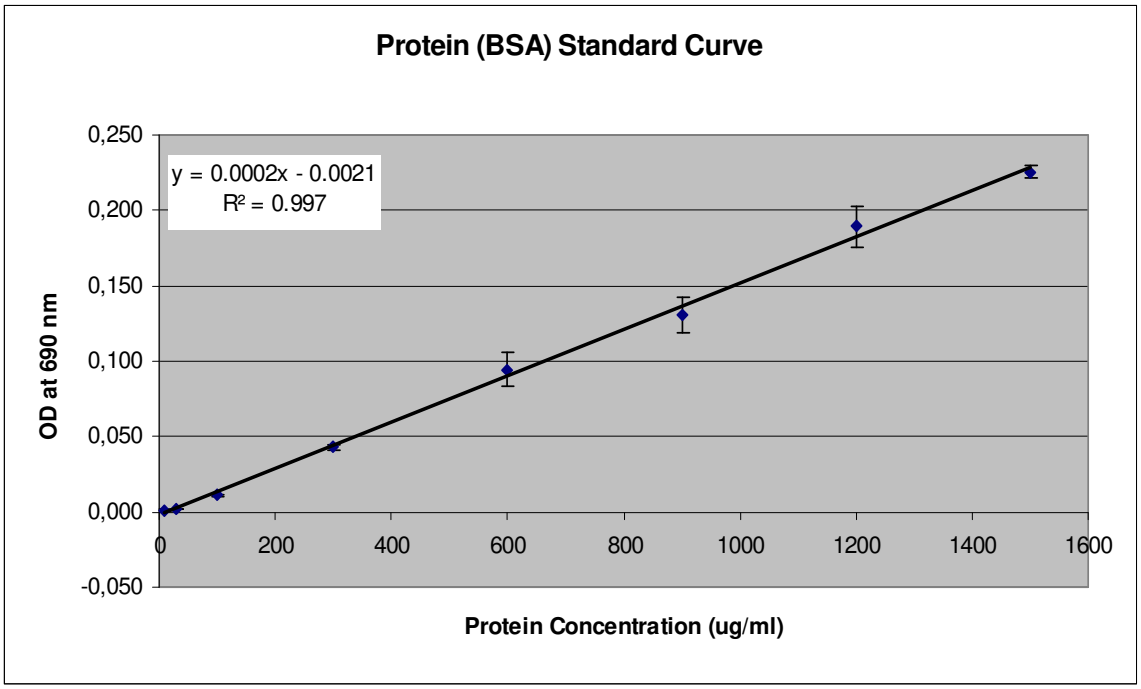
ANEXO I



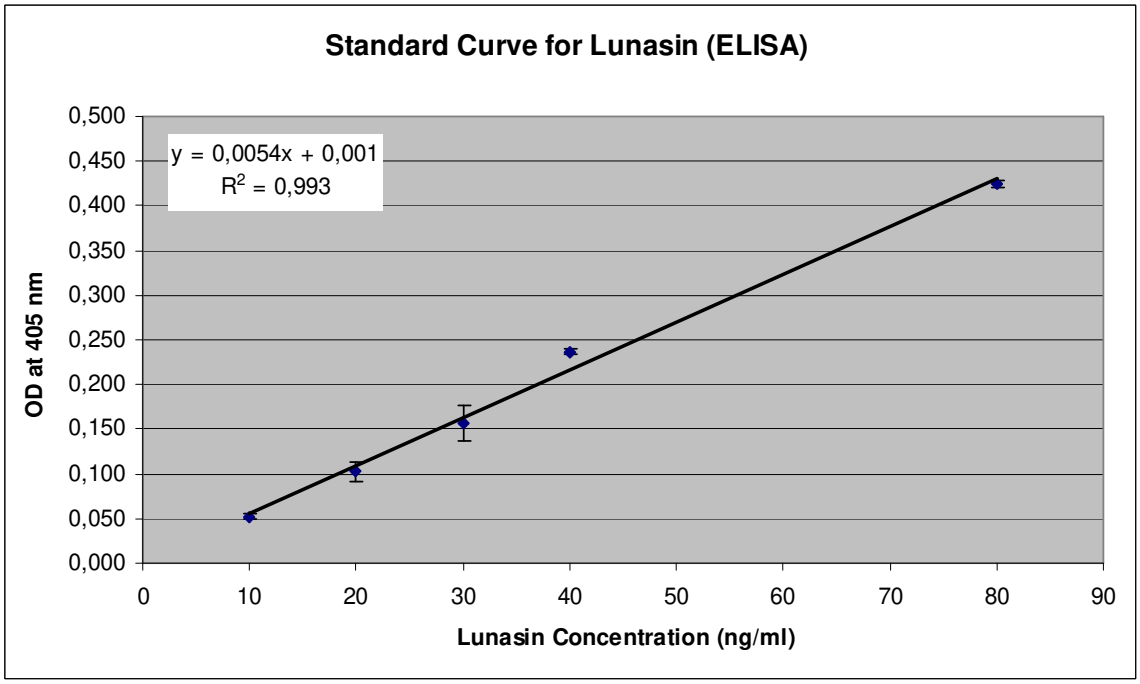
Anexo 1A. Tempo de maceração x conteúdo de umidade dos grãos de soja cultivar BRS 133 (500g de soja em 1 L).



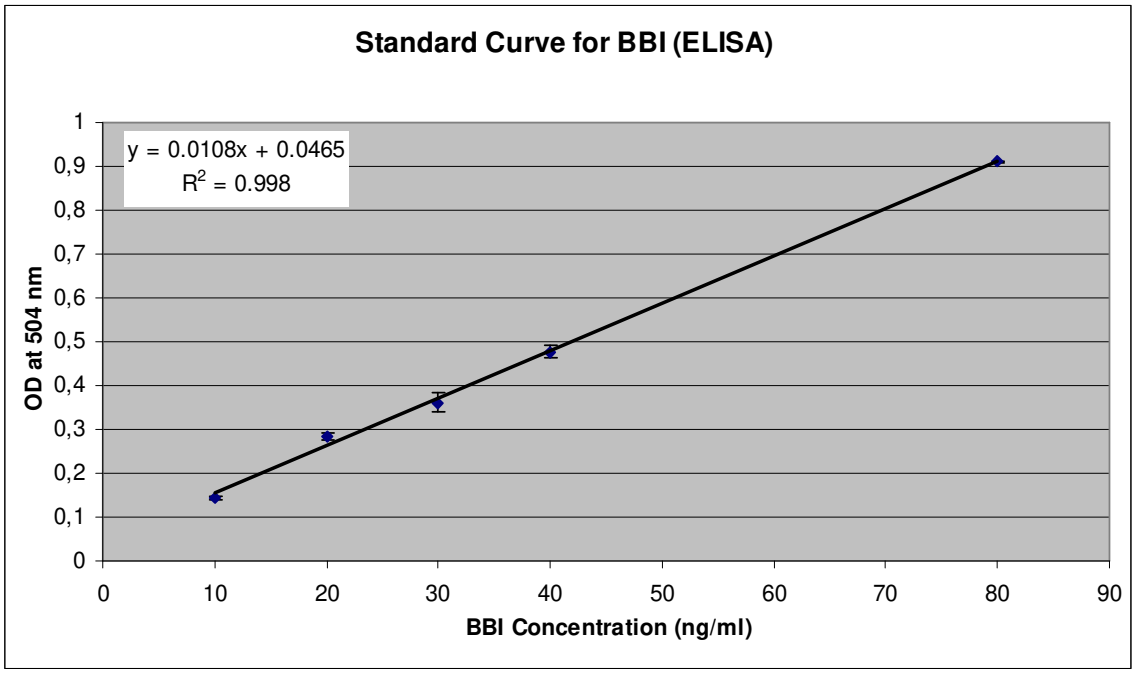
Anexo 1B. Tempo de maceração x conteúdo de umidade dos grãos de soja cultivar BRS 258 (500g de soja em 1L).



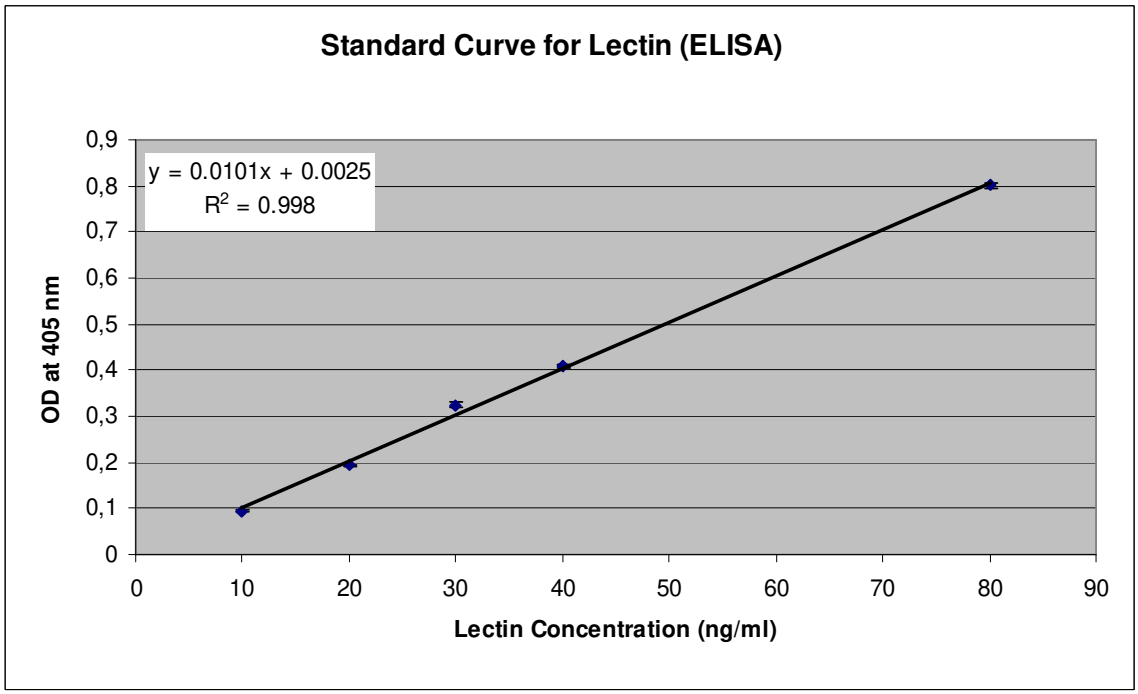
Anexo 1C. Curva padrão para concentração de proteína soluvel



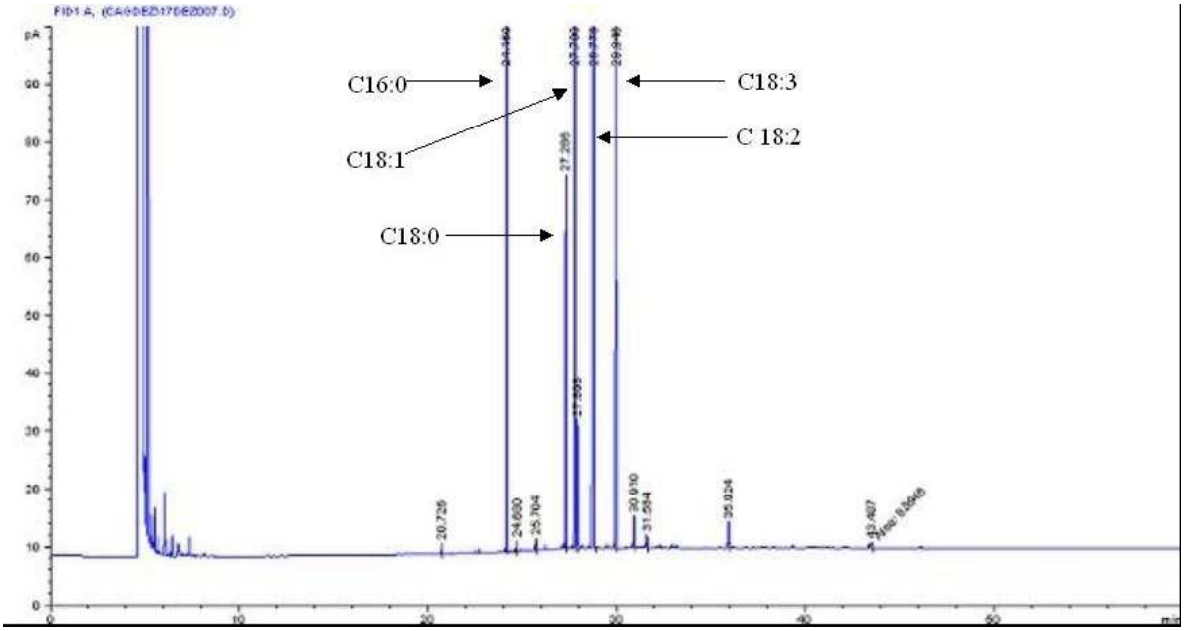
Anexo 1D. Curva padrão para concentração de lunasina.



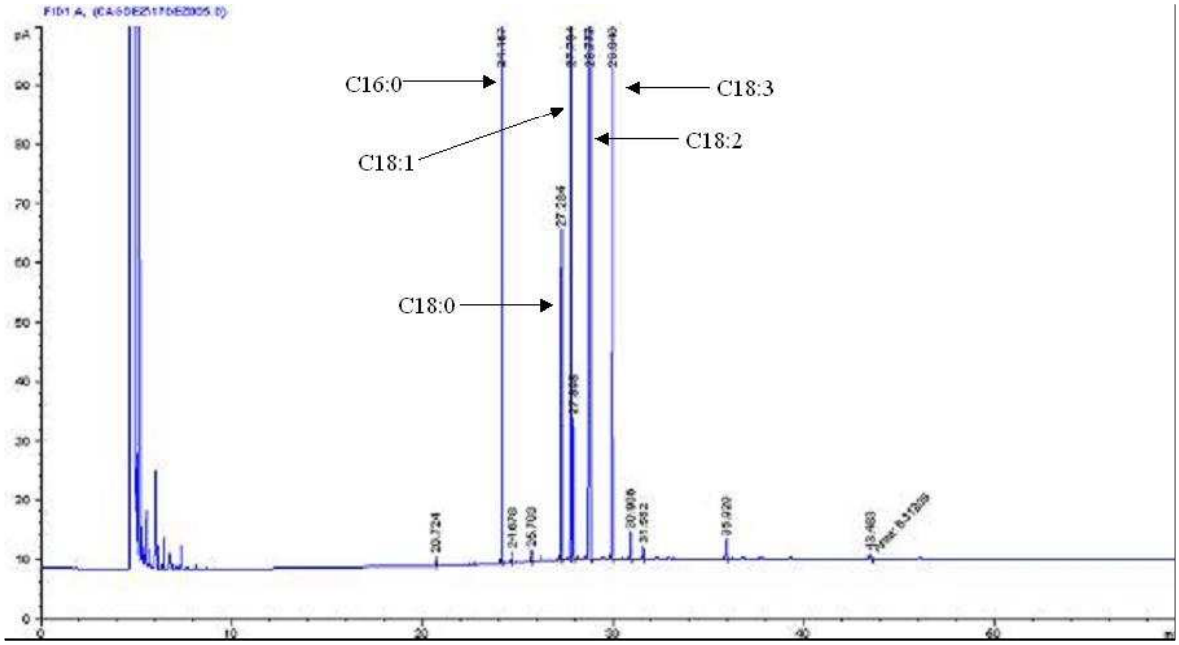
Anexo 1E. Curva padrão para concentração de BBI



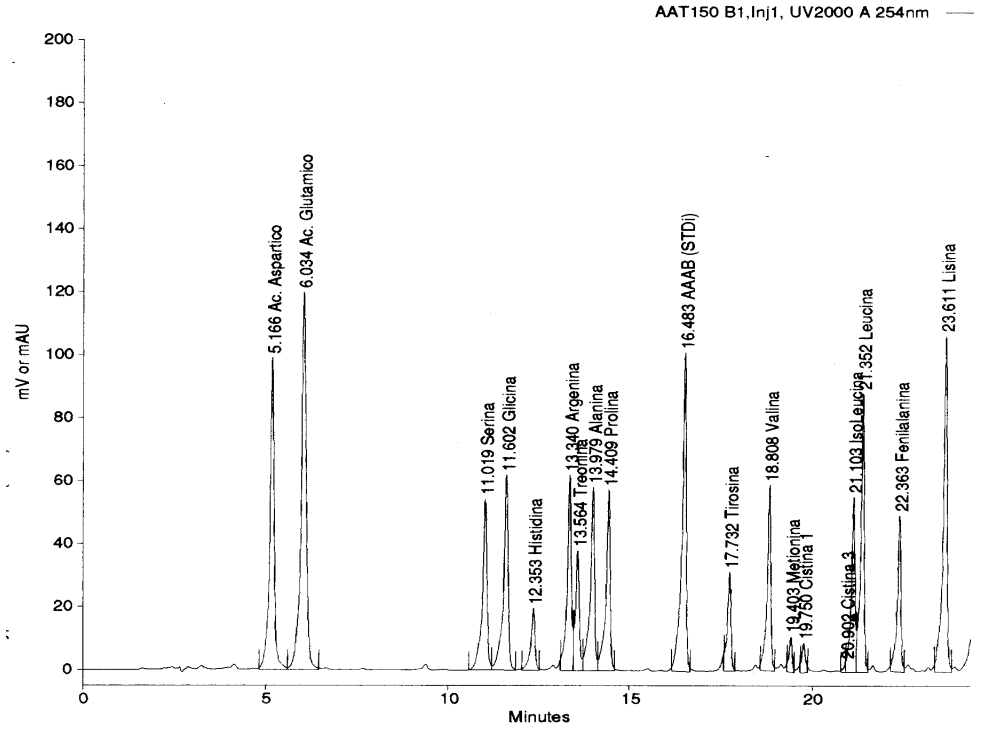
Anexo 1F. Curva padrão para concentração de lectina.



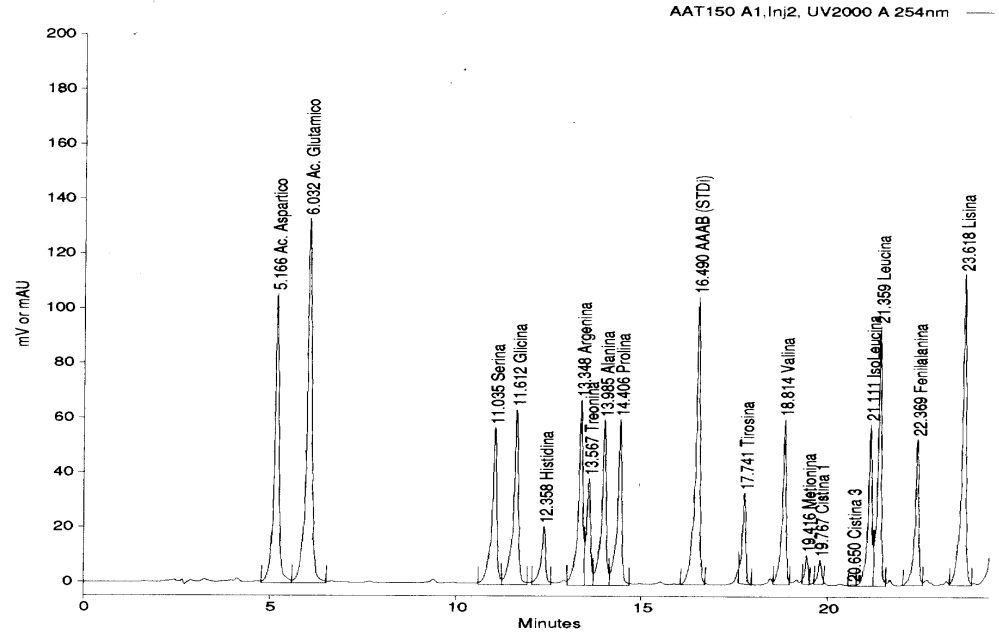
Anexo 1G. Cromatograma de ácidos graxos da cultivar BRS 133



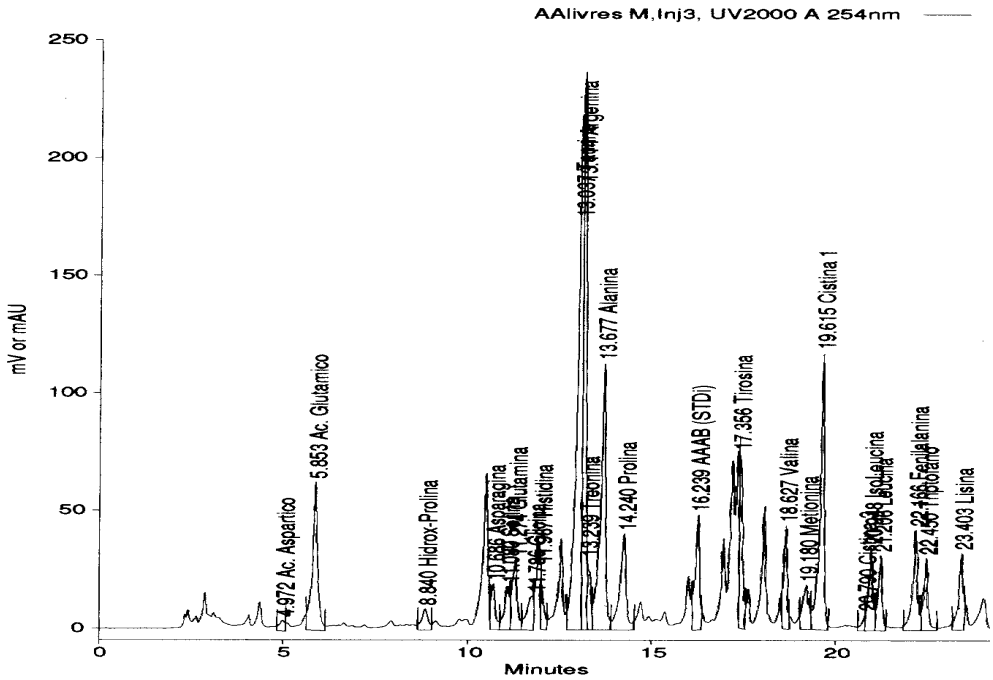
Anexo 1H. Cromatograma de ácidos graxos da cultivar BRS 258



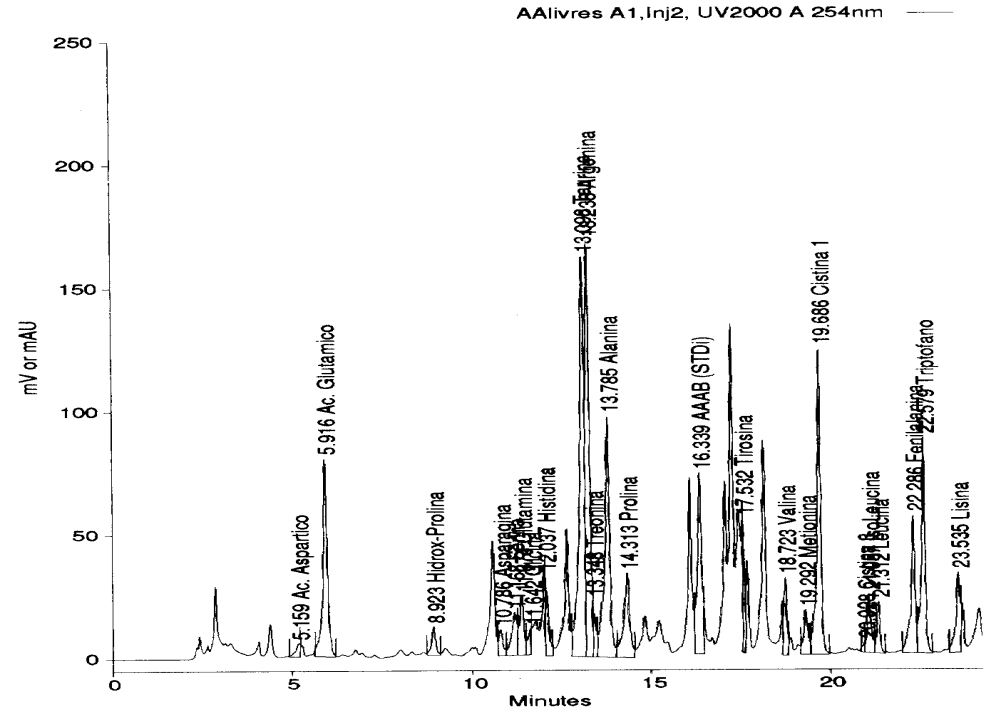
Anexo 1I. Cromatograma dos aminoácidos totais da cultivar BRS 133



Anexo 1J. Cromatograma dos aminoácidos totais da cultivar BRS 258.



Anexo 1K.Cromatograma dos aminoácidos livres da cultivar BRS 133



Anexo 1L. Cromatograma dos aminoácidos livres da cultivar BRS 258.

ANEXO II

Modelos, coeficientes de regressão, erro padrão, valores t e valores p. da farinha integral de soja germinada da cultivar BRS 133

PROTEÍNA SOLÚVEL

Modelo de regressão ajustado para proteína solúvel da cultivar BRS 133, (R^2=0,81):

Proteína solúvel (mg/g flour) **= 228,88 – 23,99 x_1 – 30,35 x_2 + 30.35 x_1x_2**

Anexo 2A. Coeficientes de regressão para a resposta proteína solúvel em farinha integral de soja germinada da cultivar BRS 133 *

	Coeficientes de regressão	Erro Padrão	t(7)	p
Media*	228,8845	6,8778	33,2787	0,0000
Tempo (L)	-23,9933	8,0649	-2,9750	0,0207
Temperatura (L)	-30,3553	8,0649	-3,7639	0,0070
Tempo x Temperatura	30,3575	11,4055	2,6616	0,0324

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2B. Anova para a resposta de proteína solúvel, na farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regressão	15663,2593	3	5221,0864	10,03
Resíduos	3642,4200	7	520,3457	
Total	19305.6793	10		

$F_{3,7;0,05}$ = 4,35

LUNASINA

Modelo de regressão ajustado para lunasina da cultivar BRS 133 (R^2=0,91):

$$\text{Lunasina (mg/g S.P.)} = \mathbf{21.08 - 2.45x_1^2 - 4.38\ x_2^2 - 4.03\ x_1x_2}$$

Anexo 2C. Coeficientes de regressão para a resposta proteína solúvel em farinha integral de soja germinada da cultivar BRS 133*.

	Coeficientes de regressão	Erro Padrão	t(7)	p
Media	21,0790	0,8965	23,5129	0,0000
Tempo (Q)	-2,4553	0,6534	-3,7576	0,0071
Temperatura (Q)	-4,3811	0,6534	-6,7048	0,0003
Tempo x Temperatura	-4,0265	0,7764	-5,1863	0,0013

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2D. Anova para a resposta de lunasina da farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regressão	181,6543	3	60,5514	25,11
Resíduos	16,8774	7	2,4110	
Total	198,5317	10		

$F_{3,7;0,05}$ = 4,35

INIBIDOR DE BOWMAN-BIRK (BBI)

Modelo de regressão ajustado para BBI da cultivar BRS 133 (R^2=0,85):

$$\text{BBI (mg/g S.P.)} = \mathbf{28.43 - 2.03\ x_1 + 3.65\ x_1\ x_2}$$

Anexo 2E. Coeficientes de regressão para a resposta BBI em farinha integral de soja germinada da cultivar BRS 133*.

	Coeficientes de regressão	Erro Padrão	t(8)	p
Media	28,42636	0,41278	68,86558	0,00000
Temperatura (L)	2,02756	0,48403	4,18894	0,00304
Tempo x Temperatura	3,65250	0,68452	5,33586	0,00070

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2F. Anova para a resposta de BBI na farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	86,2511	2	43,1255	23,01
Residuos	14,9941	8	1,8742	
Total	101,2452	10		

$F_{2,8;0,05}$=4,46

LECTINA

Modelo de regressão ajustado para lectina da cultivar BRS 133 (R^2=0,89):

$$\text{Lectina (mg/g S.P.)} = \mathbf{7,40 - 1,12x_1 + 2.05\ x_1^2 + 2.15\ x_2^2}$$

Anexo 2G. Coeficientes de regressão para a resposta lectina em farinha integral de soja germinada da cultivar BRS 133*.

	Coeficientes de regressão	Erro Padrão	t(7)	p
Media	7,3990	0,5286	13,9961	0,0000
Tempo (L)	-1,1230	0,3237	-3,4689	0,0104
Tempo (Q)	2,0543	0,3853	5,3313	0,0011
Temperatura (Q)	2,1530	0,3853	5,5876	0,0008

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2H. Anova para a resposta de lectina da farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	48,7482	3	16,2494	19,38
Residuos	5.8689	7	0,8384	
Total	54,6171	10		

$F_{3,7;0,05} = 4,35$

LIPOXIGENASE

Modelo de regressão ajustado para lipoxigenase da cultivar BRS 133 (R^2=0,93):

$$\text{Lipoxigenase (\%)} = \mathbf{4.01 - 1{,}52x_1 + 1.05\ x_1^2 - 0{,}62\ x_2^2 + 1.86\ x_1x_2}$$

Anexo 2I. Coeficientes de regressão para a resposta lipoxigenase em farinha integral de soja germinada da cultivar BRS 133*.

	Coeficientes de regressão	Erro Padrão	t(6)	p
Media	4,0100	0,4220	9,5017	0,0001
Tempo (L)	-1,5181	0,2584	-5,8739	0,0011
Tempo (Q)	1,0513	0,3076	3,4175	0,0142
Temperatura (L)	-0,6210	0,2584	-2,4028	0,0513
Temperatura (Q)	1,8663	0,3076	6,0670	0,0009

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2J. Anova para a resposta de lipoxigenase na farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	42,7490	4	10,6872	20,00
Residuos	3,2060	6	0,5343	
Total	45,9550	10		

$F_{4,6;;0,05} = 4,53$

ISOFLAVONAS TOTAIS

Modelo de regressão completo para isoflavones totais da cultivar BRS 133 (R^2=0,72):

Isoflavones totais(mg/100g)=**$369.44 - 17.60x_1 - 36.84x_2$**

Anexo 2K. Coeficientes de regressão para a resposta isoflavonas totais em farinha integral de soja germinada da cultivar BRS 133*.

	Coeficientes de regressão	Erro Padrão	t(8)	p
Media	363,8867	7,6383	48,3670	0,0000
Tempo (L)	-17,6015	8,9567	-1,9652	0,00850
Temperatura (L)	-36,8488	8,9567	-4,1141	0,0034

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2L. Anova para a resposta de isoflavonas totais, na farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	13341,1728	2	6670.5864	10,39
Residuos	5134,2654	8	641.7873	
Total	18475,4382	10		

$F_{2,8;0,05}$=4,46

AGLICONAS TOTAIS

Modelo de regressão completo para aglyconas totais da cultivar BRS 133 (R^2=0,846):

$$\text{Aglyconas totais (mg/g)} = \mathbf{26.51 + 12.69x_1 + 15.12x_2 + 19.10\ x_1x_2}$$

Anexo 2M. Coeficientes de regressão para a resposta lectina em farinha integral de soja germinada da cultivar BRS 133 * (R^2=0,846).

	Coeficientes de regressão	Erro Padrão	t(7)	p
Media	26,5109	3,2783	8,0869	0,0001
Tempo (L)	12,6866	3,8441	3,3003	0,0131
Temperatura (L)	15,1224	3,8441	3,9339	0,0056
Tempo x Temperatura	19,1000	5,4364	3,5134	0,0098

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2N. Anova para a resposta de aglyconas totaisl, na farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	4576,3349	3	1525.4449	12.92
Residuos	827,5244	7	118.2177	
Total	5403,8593	10		

$F_{3,7;;0,05} = 4,35$

SAPONINAS TOTAIS

Modelo de regressão completo para saponinas totais da cultivar BRS 133 (R^2=0,846):

TOTAL SAPONINS (mg/g) = $\mathbf{10.83 - 1.58x_1 + 0.46x_2}$

Anexo 2O. Coeficientes de regressão para a resposta lectina em farinha integral de soja germinada da cultivar BRS 133 * (R^2=0,846).

	Coeficientes de regressão	Erro Padrão	t(8)	p
Media	10,8327	0,1320	82,0588	0,0000
Tiempo (L)	1,5851	0,1548	10,2397	0,0000
Temperatura (L)	0,4600	0,1548	2,9714	0,0178

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2P. Anova para a resposta de saponinas totais, na farinha integral de soja germinada da cultivar BRS 133.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	21,7922	2	10,8961	56,84
Residuos	1,5336	8	0,1917	
Total	23,3258	10		

$F_{2,8;0,05}$ = 4,46

ANEXO III

Modelos, coeficientes de regressão, erro padrão, valores t e valores p. da farinha integral de soja germinada da cultivar BRS 258

PROTEÍNA SOLÚVEL

Modelo de regressão completo para proteína solúvel da cultivar BRS 258 (R^2=0,84):

Prot.solúvel (mg/g flour) **= 297.52 + 24.52x_1 + 11.81x_1^2 + 13.38x_2**

Anexo 3A. Coeficientes de regressão para a resposta proteína solúvel em farinha integral de soja germinada da cultivar BRS 258 *

	Coeficientes de regressão	Erro Padrão	t(7)	p
Media	297,5235	5,6763	52,4145	0.0000
Tempo (L)	24,5222	4,7773	5,1330	0,0013
Tempo (Q)	11,8176	5,4346	2,1744	0,00661
Temperature (L)	13,3832	4,7773	2,8013	0,0264

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2B. Anova para a resposta de proteína solúvel, na farinha integral de soja germinada da cultivar BRS 258.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regressão	7106,9582	3	2368.9861	12,97
Resíduos	1278,0981	7	182,5844	
Total	8385,053	10		

$F_{3,7;;0,05}$ = 4,35

LUNASINA

Modelo de regressão ajustado para lunasina da cultivar BRS 258 (R^2=0,89):

$$\text{Lunasina (mg/g S.P.)} = \mathbf{11.76 - 3.55x_1 - 3.36x_2^2}$$

Anexo 2C. Coeficientes de regressão para a resposta proteína solúvel em farinha integral de soja germinada da cultivar BRS 258*.

	Coeficientes de regressão	Erro Padrão	t(8)	p
Media	11,7652	0,6773	17,3696	0,0000
Tempo (L)	-3,5555	0,5701	-6,2369	0,0002
Temperatura (Q)	-3,3684	0,6485	-5,1940	0,0008

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2D. Anova para a resposta de lunasina da farinha integral de soja germinada da cultivar BRS 258.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regressão	171,2700	2	85,6350	32,94
Resíduos	20,7987	8	2,5998	
Total	192,0687	10		

$F_{2,8;0,05} = 4,46$

INIBIDOR DE BOWMAN-BIRK (BBI)

Modelo de regressão ajustado para BBI da cultivar BRS 258 (R^2=0,95):

$$\text{BBI (mg/g S.P.)} = \mathbf{17.46 - 2.35x_1 + 1.57x_1^2 - 1.13\ x_2 + 1.15\ x_2^2}$$

Anexo 2E. Coeficientes de regressão para a resposta BBI em farinha integral de soja germinada da cultivar BRS 258*.

	Coeficientes de regressão	Erro Padrão	t(6)	p
Media	17,4600	0,4450	39,2372	0,0000
Tempo (L)	-2,3459	0,2725	-8,6090	0,0001
Tempo (Q)	1,5756	0,3243	4,8580	0,0028
Temperatura (L)	-1,1290	0,2725	-4,1432	0,0061
Temperatura (Q)	1,1556	0,3243	3,5630	0,0119

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2F. Anova para a resposta de BBI na farinha integral de soja germinada da cultivar BRS 258.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	71,2055	4	17.8013	29,97
Residuos	3,5642	6	0,5940	
Total	74,7697	10		

$F_{4,6;0,05} = 4,53$

LECTINA

Modelo de regressão ajustado para lectina da cultivar BRS 258 (R^2=0,92):

$$\text{Lectina (mg/g S.P.)} = \mathbf{20.28 - 5.17\ x_1 - 4.02\ x_2^2}$$

Anexo 2G. Coeficientes de regressão para a resposta lectina em farinha integral de soja germinada da cultivar BRS 258*.

	Coeficientes de regressão	Erro Padrão	t(8)	p
Media	20,2791	0,7911	25,6329	0,0000
Tempo (L)	-5,1685	0,6658	-7,7624	0,0001
Temperatura (Q)	-4,0162	0,7575	-5,3023	0,0007

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2H. Anova para a resposta de lectina da farinha integral de soja germinada da cultivar BRS 258.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	313,4175	2	156,,70875	44,18
Residuos	28,3738	8	3,5467	
Total	341,7913	10		

$F_{2,8;0,05} = 4,46$

LIPOXIGENASE

Modelo de regressão ajustado para lipoxigenase da cultivar BRS 258 (R^2=0,98):

$$\text{Lipoxigenase (\%)} = \mathbf{3.42 - 1{,}59\ x_1 + 1{,}52\ x_1^2 - 1.18\ x_2 + 0.75\ x_2^2}$$

Anexo 2I. Coeficientes de regressão para a resposta lipoxigenase em farinha integral de soja germinada da cultivar BRS 258*.

	Coeficientes de regressão	Erro Padrão	t(6)	p
Media	3,4233	0,2111	16,2137	0,0000
Tempo (L)	-1,5926	0,1293	-12,3176	0,0000
Tempo (Q)	1,5165	0,1539	9,8540	0,0001
Temperatura (L)	-1,1811	0,1293	-9,1347	0,0001
Temperatura (Q)	0,7515	0,1539	4,8830	0,0028

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2J. Anova para a resposta de lipoxigenase na farinha integral de soja germinada da cultivar BRS 258.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	45,0136	4	11,2534	84,17
Residuos	0,8024	6	0,1337	
Total	45,8160	10		

$F_{4,6;0,05} = 4{,}53$

ISOFLAVONAS TOTAIS

Modelo de regressão completo para isoflavones totais da cultivar BRS 258 (R^2=0,93):

Isoflavones totais(mg/100g)= $\mathbf{241.78 + 11.63x_1^2 - 14.29x_2 + 11.67x_2^2 - 16.05x_1x_2}$

Anexo 2K. Coeficientes de regressão para a resposta isoflavonas totais em farinha integral de soja germinada da cultivar BRS 258*.

	Coeficientes de regressão	Erro Padrão	t(6)	p
Media	241,7833	3,8814	62,2922	0,0000
Tempo (Q)	11,6321	2,8291	4,1116	0,0063
Temperatura (L)	-14,2941	2,3769	-6,0138	0,0010
Temperatura (Q)	11,6721	2,8291	4,1258	0,0062
Tempo x Temperatura	-16,0550	3,3614	-4,7763	0,0031

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2L. Anova para a resposta de isoflavonas totais, na farinha integral de soja germinada da cultivar BRS 258.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	3850,5310	4	962,6327	21,30
Residuos	271,1800	6	45,1966	
Total	4121,7110	10		

$F_{4,6;0,05}$ = 4,53

AGLICONAS TOTAIS

Modelo de regressão completo para aglyconas totais da cultivar BRS 258 (R^2=0,92):

$$\text{Aglyconas totais (mg/g)} = \mathbf{22,62 + 6,27\ x_1 + 12,02\ x_2 + 15,31\ x_1x_2}$$

Anexo 2M. Coeficientes de regressão para a resposta lectina em farinha integral de soja germinada da cultivar BRS 258 * (R^2=0,846).

	Coeficientes de regressão	Erro Padrão	t(7)	p
Media	22,6164	1,6855	13,4182	0,0000
Tempo (L)	6,2741	1,9764	3,1745	0,0156
Temperatura (L)	12,0214	1,9764	6,0824	0,0005
Tempo x Temperatura	15,3050	2,7951	5,4757	0,0009

*Termos estatisticamente significativos ao nível de 5% de significância.

Anexo 2N. Anova para a resposta de aglyconas totaisl, na farinha integral de soja germinada da cultivar BRS 258.

Fontes de variação	Soma de quadrados	Graus de liberdade	Quadrado médio	F cal
Regresão	2408,0003	3	802,6667	25,68
Residuos	218,7482	7	31,2497	
Total	2626,7485	10		

$F_{3,7;0,05}$ = 4,35

SAPONINAS TOTAIS

Anexo 2O. Coeficientes de regressão para a resposta lectina em farinha integral de soja germinada da cultivar BRS 258*.

	Coeficientes de regressão	Erro Padrão	t(5)	p
Media	7,7200	2,7864	2,7706	0,0393
Tempo (L)	2,3953	1,7063	1,4038	0,2193
Tempo (Q)	0,8988	2,0309	0,4425	0,6766
Temperatura (L)	1,6969	1,7063	0,9945	0,3657
Temperatura (Q)	0,8012	2,0309	0,3945	0,7095
Tempo x Temperatura	3,4175	2,4131	1,4162	0,2159

*Termos estatisticamente significativos ao nível de 5% de significância.

As saponinas totais no caso da cultivar BRS 258 não apresentaram modelo signitificativo ao nivel de 5% de significância (R^2=0,51).