TIAGO FIDEMANN

OTIMIZAÇÃO DAS CONDIÇÕES DE CULTIVO DE CALOS DE *Capsicum baccatum* var. pendulum

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Dissertação apresentada à Faculdade de Ciências e Letras de Assis – UNESP – Universidade Estadual Paulista para obtenção do título de Mestre em Biociências (Área de Conhecimento: Aplicação da Diversidade Biológica)

Orientador: Dr. Eutimio Gustavo Fernández Núñez
Co-Orientador(a): Dra Mônica Rosa Bertão
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RESUMO

O estresse oxidativo e as inflamações estão associados a muitas doenças como diabetes, câncer, doenças cardiovasculares e neurodegenerativas, incluindo a aterosclerose, causando preocupação em todo o mundo. Como alternativa, as plantas podem desempenhar um papel chave no combate a essas desordens devido suas propriedades farmacológicas que são relacionadas a uma ampla gama de compostos com atividade antioxidante, entre eles, os compostos polifenólicos. Nesse contexto as pimentas podem ser uma alternativa para a obtenção desses compostos. Porém, a concentração desses metabólitos secundários é baixa e afetada por diversos fatores abióticos. Como alternativa para contornar esse problema foram utilizadas metodologias relacionadas com as culturas *in vitro* de células e de tecidos vegetais. O presente trabalho teve como objetivo padronizar a obtenção de explantes (Capítulo 1) a serem utilizados em uma cultura de calos otimizada através de uma abordagem sistêmica (Capítulo 2). No capítulo 1, por meio de um delineamento experimental fatorial completoadois níveis (2^4) determinou-se numericamente o efeito de quatro fatores [O tipo de solução para condicionamento osmótico (água e solução aquosa de KNO_3 a 1%), meio germinativo (ágar e ágar + ácido giberélico a 1,88 $mg.L^{-1}$), tempo (15 e 30 dias) e dois genótipos (Pitanga e Cambuci)] nas taxas de surgimento de cotilédones e na germinação. As respostas dos genótipos a solução de condicionamento osmótico e ao meio de germinação foram diferentes. A melhor combinação de tratamentos para germinação *in vitro* e desenvolvimento de plântulas para Pitanga e Cambuci foram água + ágar e água + ágar-ácido giberélico (GA_3), respectivamente. No capítulo 2, objetivando-se otimizar a etapa de cultura de calos da pimenta Cambuci para maior produção de biomassa e metabólitos secundários foram avaliados a origem do explante, o hormônio de crescimento e a concentração do mesmo através de um delineamento fatorial multiníveis. Os valores obtidos nas condições otimizadas foram: massa de calo (225,03 mg), atividade antioxidante (35,95%), fenóis totais (11,48mg de GAE / g DE) e flavonóides (15,92 mg de RU / g DE).

Palavras-chave: Germinação *in vitro*. Cultivo de calos. Produção de metabólitos secundários através de bioprocessos. *Capsicum baccatum* var. pendulum. Modelagem estatística.

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ABSTRACT

The oxidative stress and inflammations are associated with many diseases such as diabetes, cancer, cardiovascular and neurodegenerative diseases including atherosclerosis causing concern worldwide. As an alternative, the plants can play a key role due to their pharmacological properties against these disorders; these attributes are most of the times associated with the presence of polyphenolic compounds. In this context, peppers can be an alternative to obtain these compounds. However, the presence of secondary metabolites is low and affected by several abiotic factors. As an alternative to circumvent this problem, methodologies related to *in vitro* cultures of plant cells and tissues were applied. The objective of the present work was to standardize the extraction of explants (Chapter 1) to be used in a callus culture optimized through a systemic approach (Chapter 2). In Chapter 1, through a two-level full factorial experimental design (2^4) was numerically determined the effect of four factors [The solution type for osmotic conditioning (water and 1% KNO_3 aqueous solution), germination medium (agar and agar + gibberellic acid at 1.88 $mg.L^{-1}$, GA_3), post-sowing time (15 and 30 days) and two genotypes (Pitanga and Cambuci)] in cotyledon emergence and germination rates. The genotypes responses to the osmotic conditioning solution and to the germination medium were different. The optimal combination of treatments for *in vitro* germination and development of seedlings for Pitanga and Cambuci was water + agar and water + agar-GA_3, respectively. In Chapter 2, aiming to optimize systemically the Cambuci pepper callus culture stage for higher biomass and secondary metabolites productionwere evaluated explant’s origin, plant growth regulator type and their concentrations through a multilevel factorial design. The values obtained in the optimized conditions were: callus mass (225.03 mg), antioxidant activity (35.95%), total phenols (11.48mg of GAE/g DE) and flavonoids (15.92 mg of RU/g DE).

Keywords: *In vitro* germination. Callus culture. Secondary metabolites production through bioprocesses. *Capsicum baccatum* var. pendulum. Statistical modeling.

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INTRODUÇÃO GERAL

Capsicum baccatum

As pimentas e pimentões do gênero *Capsicum* pertencem à família Solanaceae são originárias da América Central e América do Sul, tendo centenas de variedades com frutos que variam em tamanho, formato, cor e sabor. Dentro desse contexto cinco espécies se destacam: *Capsicum baccatum*, *Capsicum annuum*, *Capsicum chinense*, *Capsicum frutescens* e *Capsicum pubescens* (ZIMMER et al., 2012). Apresentam expressiva importância econômica e social para o agronegócio mundial, associada a seu alto aproveitamento na culinária para temperos. Possuem altos valores vitamínicos e são fonte de antioxidantes naturais. Entre os principais componentes químicos das pimentas destacam-se os caretonóides, o ácido ascórbico, os tocoferóis, a vitamina A e os capsaicinóides (Figura 1), além de minerais como molibdênio, manganês e potássio, cujas concentrações podem variar com o grau de maturação e o genótipo (KOTHARI et al., 2010; PINTO et al., 2013).

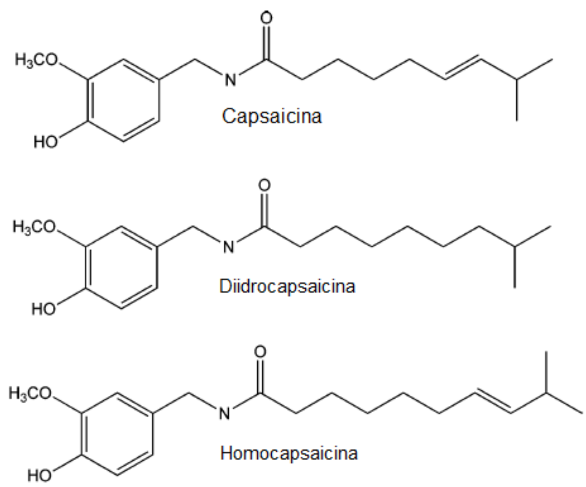

Figura 1 – Estruturas químicas de alguns capsaicinóides naturais. Fonte: adaptado de DAVIS et al., (2007).

A culinária brasileira faz amplo consumo das diferentes variedades de *Capsicum*, entre elas, a *Capsicum baccatum* var. pendulum (Figura 2). Recentemente tem sido demonstrada atividade anti-inflamatória e antioxidante dessas pimentas devido aos seus compostos bioativos, entre eles, os compostos fenólicos (BERTÃO et al., 2016; ZIMMER et al., 2012).

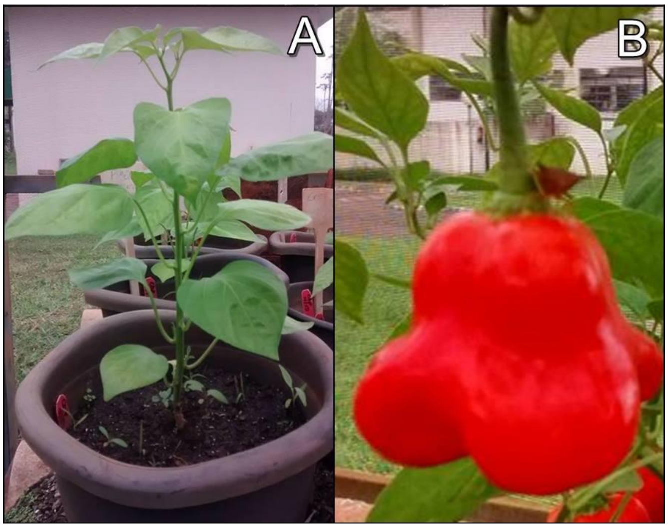

Figura 2 – *Capsicum baccatum* L. var pendulum (A). Fruto maduro de *Capsicum baccatum* L. var pendulum (B)

Estresse oxidativo

O estado pró-oxidante (estresse oxidativo) tem sido relacionado a diversas doenças inflamatórias, cardiovasculares e diferentes formas de câncer (MATKOWSKI, 2008; NIKI, 2010). Isso ocorre quando há um desequilíbrio entre a produção de radicais livres e sua neutralização pelos sistemas antioxidantes, sendo extremamente nocivo aos componentes celulares e indivíduos como um todo (BARREIROS et al., 2006). Os radicais livres podem ser definidos como qualquer espécie, de existência independente, que contém um ou mais elétrons desemparelhados, fato que os torna instáveis e altamente reativos (HALLIWELL e CROSS, 1994). Podem ser gerados tanto por fontes endógenas como exógenas. As fontes endógenas estão relacionadas com reações metabólicas como a oxidação nas mitocôndrias durante a respiração celular, a fagocitose durante o processo inflamatório e a atividade de enzimas como oxidases, lipoxigenases e peroxidases. As fontes exógenas são a poluição do ar, a utilização de medicamentos, pesticidas, tabagismo, dieta alimentar e radiação ultravioleta. Em condições normais existe um equilíbrio entre a produção de radicais livres e sua neutralização pelos sistemas antioxidantes. Porém, quando esse equilíbrio é afetado pela produção excessiva de radicais ou pela deficiência dos sistemas gera-se o estresse oxidativo (BARREIROS et al., 2006; MARTELLI e NUNES, 2014).

Os radicais livres envolvidos neste processo de estresse oxidativo podem estar relacionados com duas vias de oxidação: as espécies reativas de oxigênio (ERO) e a de nitrogênio (ERN). As ERO (Figura 3) estão divididas em dois grupos, os radicalares: superóxido ($O_2^{-\cdot}$), hidroperoxila ($HO^{\cdot}_2$), hidroxila ($OH^{\cdot}$), oxigênio singlete ($^1O_2$); e os não radicalares: ácido hipocloroso e peróxido de hidrogênio (H_2O_2). Entre as ERN encontram-se o radical óxido nítrico ($NO^{\cdot}$), nitritos (NO_2^-), nitratos (NO_3^-), óxido nitroso (N_2O_3), ácido nitroso (HNO_2) e peroxinitrito ($ONOO^-$) que podem desencadear reações de oxidação nos ácidos graxos da membrana lipoprotéica, denominada de peroxidação lipídica, afetando a integridade estrutural e funcional da membrana celular e alterando sua fluidez e permeabilidade (MARTELLI e NUNES, 2014; TEIXEIRA, 2013).

Além disso, os produtos da oxidação dos lipídios da membrana podem causar alterações em certas funções celulares, modificações nas proteínas celulares, resultando em sua fragmentação, agregação e, em certos casos, ativação ou inativação de certas enzimas devido à reação dos radicais livres com aminoácidos constituintes da cadeia polipeptídica (BARREIROS et al., 2006;SOARES, 2002; TEIXEIRA, 2013).

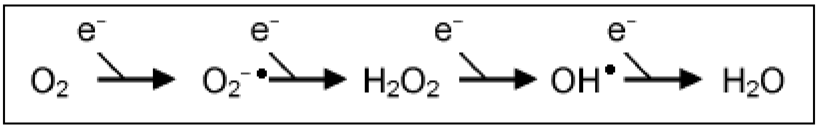

Figura 3 – Formação de algumas das espécies reativas de oxigênio (ERO) durante o transporte de elétrons na mitocôndria. O superóxido ($O_2^{-\cdot}$), peróxido de hidrogênio (H_2O_2) e radicais hidroxil ($OH^{\cdot}$) são formados como resultado da sucessiva transferência de elétrons isolados. A citocromo C oxidase transfere um total de quatro elétrons com extrema eficiência, mesmo assim, de 1-2% dos elétrons são constantemente perdidos dentro da célula como ERO potencialmente tóxicos. Fonte: HIRATA et al., (2004).

Diante desses estudos que relacionam diversas doenças com o estresse oxidativo torna-se importante o combate desses radicais livres pela absorção e reação de compostos com características sequestradoras de radicais livres, sendo estes representados por substâncias com capacidade antioxidante. As substâncias antioxidantes podem ser definidas como aquelas, que mesmo em baixas concentrações, são capazes de atrasar ou inibir as taxas de oxidação (BARBOSA et al., 2010; SHAMI e MOREIRA, 2004). Tais substâncias têm a capacidade de inibir os processos oxidativos pelo bloqueio do início da oxidação, por meio da remoção de espécies reativas, ou ainda interrompendo a cadeia de reação, doando elétrons ou hidrogênio, convertendo as espécies radicalares em moléculas termodinamicamente estáveis.

Alguns antioxidantes são encontrados no organismo, conhecidos como antioxidantes endógenos, e podem ser divididos em enzimáticos (superóxido dismutase, catalase e, glutationa peroxidase) e não enzimáticos (glutationa, bilirubina e ácido lipóico) (ANGELO e JORGE, 2007; BARBOSA et al., 2010; SILVA et al., 2010). Além do sistema antioxidante endógeno o organismo utiliza ainda substâncias antioxidantes obtidas da dieta, como ácido ascórbico, α-tocoferol, β-caroteno e compostos fenólicos, entre eles, os flavonóides (Figuras 4 e 5) que servem como um importante complemento no sistema de defesa humano contra os radicais livres (PEREIRA et al., 2009; STRINGHETA et al., 2006).

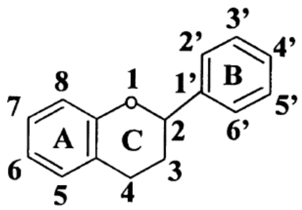

Figura 4 – Estrutura nuclear dos flavonóides. Fonte: HEIM et al., (2002).

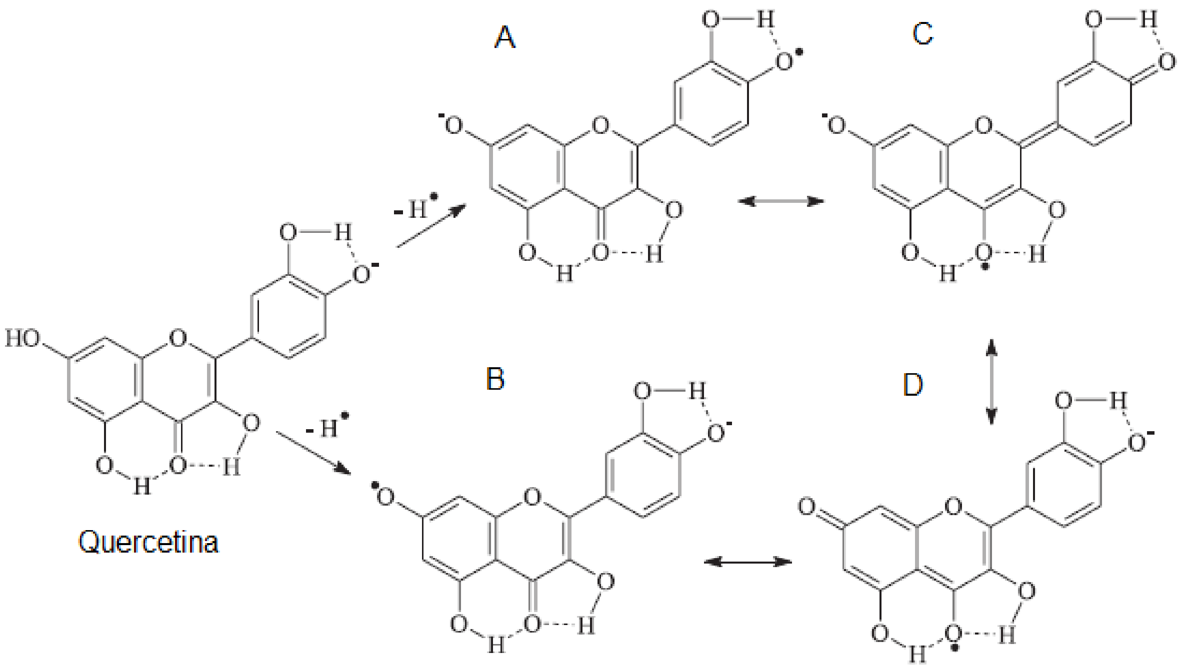

Figura 5 – Quercetina parcialmente ionizada em C-4'. A doação do H radicalar para o radical livre ocorre principalmente nas posições C-4' (A) e C-7 (B). Ambos os radicais livres formados podem ter seu elétron deslocalizado pela estrutura, com maior estabilidade para os radicais A, C e D, devido à estabilização resultante das ligaçõesde hidrogênio. Fonte: Adaptado de BARREIROS et al., (2006).

A partir disso observa-se a importância de estudos recentes que têm demonstrado capacidade antioxidante, anti-inflamatória e fotoprotetora de certas plantas e alimentos que formam as bases de sistemas tradicionais de medicina milenares (PETRUSSA et al., 2013; RAMKISSOON et al., 2013; RANILLA et. al., 2010).

Metabólitos secundários e sua obtenção por cultura de calos e em suspensão de células de plantas

Metabólitos secundários das plantas são compostos pouco abundantes, com uma frequência inferior a 1% da massa seca, de baixa massa molecular, que desempenham um importante papel na adaptação das plantas aos seus ambientes, porém não afetam diretamente seu desenvolvimento e crescimento (FUMAGALI et al., 2008; WILSON e ROBERTS, 2012). Devido à sua grande atividade biológica esses metabólitos têm sido aplicados na medicina tradicional há muitos anos. Podem atuar como antibióticos, antifúngicos, antivirais e antioxidantes e são também comumente utilizados como inseticidas, corantes, saborizantes, cosméticos ou fragrâncias (AOYAGI, 2011; FUMAGALI et al., 2008).

Em geral a síntese dos metabólitos secundários nas plantas é afetada por diversas condições ambientais como sazonalidade, índice pluviométrico, altitude e temperatura que alteram os níveis de metabólitos, dificultando a obtenção dos mesmos de modo reproduzível (GOBBO-NETO e LOPES, 2007).

Para contornar esse problema a cultura de células de plantas em suspensão tem sido utilizada na produção de diferentes metabólitos secundários. Pode-se citar a produção de digoxina, morfina, paclitaxel, artemisina entre outros; sendo alguns destes produzidos em escala comercial como, por exemplo, o paclitaxel (taxol, droga anticâncer). Desta maneira a utilização de células de plantas pode constituir uma alternativa economicamente viável para obter metabólitos secundários de relativa alta complexidade química e alto valor agregado (NAILL et al., 2012; DORAN, 2010).

O cultivo de células vegetais pode ser realizado a partir de qualquer fragmento da planta (explante), que são desinfectados utilizando um tratamento químico exógeno, para depois colocá-los em meio de cultura sólido formulado com os hormônios de crescimento e nutrientes necessários para cada espécie. Com uma correta formulação da composição dos meios, após 2 à 6 semanas, os explante proliferam em calos, massa de células indiferenciadas (figura 6), que podem ser submetidos a um processo de triagem para o produto ou os produtos de interesse. Finalmente, são isolados, fragmentados e transferidos a um meio líquido para gerar uma cultura de células em suspensão (CARVALHO e VIDAL, 2003; MUSTAFA et al*.,* 2011).

Figura 6 – Calos originados de cotilédones de *C. baccatum* var. pendulum

Parâmetros de operação críticos associados às etapas de cultura de calos e cultivos em suspensão de células vegetais

Entre os parâmetros operacionais mais importantes a serem otimizados na etapa de cultivo de calos visando alta produtividade de biomassa e metabólitos de interesse, encontram-se o explante (principalmente tecidos meristemáticos: ex. gemas, folhas jovens) e a concentração de hormônio no meio de cultura. Também deve ser definido um protocolo de desinfecção dos explantes, tendo como variáveis fundamentais a seleção do desinfetante, a concentração do mesmo e o tempo de imersão do explante na solução desinfetante (FUMAGALI et al., 2008; MUSTAFA et al., 2011; GODOY-HERNÁNDEZ e VÁZQUEZ-FLOTA, 2006).

Por outro lado, quando é pretendida a otimização da cultura de células vegetais em suspensão, outros parâmetros de operação como luminosidade, temperatura, velocidade de agitação, concentração de oxigênio dissolvido, composição do meio de cultura, devem ser considerados. Quando as culturas em suspensão são realizadas em frascos erlenmeyers, recomenda-se trabalhar com um volume de suspensão celular equivalente entre 20 e 40 % do volume do frasco, velocidade de agitação entre 90-125 rpm e a uma temperatura próxima a temperatura ambiente (24-25 ^{o}C). Entretanto, entre os componentes que necessitam ter suas concentrações estão as fontes de carbono, macroelementos (Mg, Ca, P, S, N, K), microelementos (Fe, Cu, Mn, Co, Mo, B, I, Ni, Cl, Al), vitaminas e hormônios de crescimento (auxinas ou citocininas) em concentrações na faixa de 10^{-7} a 10^{-5} $mol.L^{-1}$. O pH do meio de cultura é normalmente ajustado entre 5,75 e 5,85 antes da esterilização do meio. De maneira geral a aeração é outro fator crítico na cultura de células vegetais em suspensão, mas o mesmo só é rigorosamente controlado quando a cultura é realizada em biorreatores. Geralmente as concentrações críticas de oxigênio dissolvido para este tipo de células encontram-se entre 15-20% de saturação. Entretanto, este parâmetro deve ser otimizado para cada sistema em particular (KIERAN et al., 1997; MUSTAFA et al., 2011; WILSON e ROBERTS, 2012).

Nesse contexto o presente estudo teve como objetivo a obtenção de explantes adequados germinados *in vitro* como alternativa aos tradicionais métodos de desinfecção para o desenvolvimento e otimização de cultura de calos de pimenta Cambuci – *Capsicum baccatum* L. var. pendulum buscando uma relação entre maior rendimento de biomassa, maior atividade antioxidante e maior teor de flavonóides e compostos fenólicos.

Para a confecção dos capítulos a seguir foram utilizadas as normas dos periódicos American International Journal of Biology (Capítulo 1) e Biotechnology Progress (Capítulo 2).

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CAPÍTULO 1 - Optimizing *in vitro* germination of *Capsicum baccatum* L. seeds through a multifactorial experimental design

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1.1 Abstract

The *Capsicum* genus comprises hundreds of varieties with fruits that differ widely in shape, size, color and flavor. The Brazilian cuisine consumes *Capsicum baccatum* L. (pepper) extensively and few reports about chemical composition and biological properties about this pepper were published. The present study determined four factors numeric effects on germination *in vitro* of this specie, among them, the solution type for osmotic conditioning (water and 1% KNO_3 aqueous solution), germination medium (agar and agar + gibberellic acid at 1.88 mgL^{-1}), post-sowing time (15 and 30 days) and two genotypes (Pitanga and Cambuci) on two germination parameters (germination and cotyledon emerging rates), as first step for establishing a cell suspension culture to produce secondary metabolites. The methodology was a two-level full factorial experimental design (2^4). The results allowed polynomial equations definition which describes the germination phenomena as a function of the four factors under study. The genotype responses to osmotic conditioning and germination medium were different. The optimal combination of treatments for *in vitro* germination and development of seedlings for Pitanga and Cambuci was water + agar and water + agar-GA_3, respectively.

Keywords: Peppers, experimental design, gibberellic acid, potassium nitrate, plant cell culture.

1.2 Introduction

The *Capsicum* genus (Solanaceae) includes hundreds of varieties with fruits that differ widely in shape, size, color and flavor. Native from Central and South America, the *Capsicum* peppers have an important economic value (Manzur et al., 2013; Zimmer et al., 2012) being used as spice and condiment in cooking around the world. Have a high nutritive value besides being a rich source of vitamins C (ascorbic acid), A (β-carotene and provitamin A), B2 (riboflavin), B3 (niacin), E, K and B6, along with minerals like molybdenum, manganese and potassium (Kothari et al., 2010). Brazilian cuisine consumes different varieties of *Capsicum baccatum* L. extensively and few reports about chemical composition and biological properties on this pepper have been published. Recently, antioxidant and anti-inflammatory activities associated to its phenolic compounds and flavonoids have been demonstrated (Zimmer et al., 2012; Bertão et al., 2016). Nevertheless, in many cases the natural availability of these compounds is low and the plant extraction methods require using some part or the entire plant. These practices are not sustainable, even more, when the plant is rare or endangered. The *in vitro* culture techniques can solve these drawbacks (Matkowski, 2008).

The plant cell and tissue culture is an essential tool for basic and applied academic studies as well as in industrial applications including several cultivation methodologies under sterile conditions such as seed germination, micropropagation, callus and cell suspension culture (Wilson and Roberts, 2012). *In vitro* propagation methods allow the selective, rapid and effective production of secondary metabolites avoiding seasonal, soil, geographical constrains as well as pests and diseases (Costa et al., 2013).

Micropropagation is one of the most important areas where *in vitro* culture has played a commercially remarkable role. *In vitro* propagation technologies when well-developed and/or adapted are critical for genetic resources and genetic improvement of the species conservation programs. A large number of important plants from horticultural, floricultural and silvicultural have been successfully propagated. Among the most important *in vitro* technique applications, is the micropropagation of medicinal plants, species threatened and plants producing spices, flavoring and coloring substance (Matkowski, 2008; Purohit, 2013).

Recent research on *Capsicum* cell culture also showed the great potential of *in vitro* cultivation techniques for understanding the cellular and molecular processes involved in the expression and production of metabolites of commercial interest (Kehie et al., 2015).

In vitro seed germination is a decisive step for the success of numerous *in vitro* culture procedures and often allows a higher germinability than in nurseries, greenhouses or field, probably because *in vitro* conditions are more suitable for germination processes and early seedling development. *In vitro* seed germination protocols are frequently used to produce sterile seedlings, which are source of shoot tips and stalk as explants for micropropagation (Kothari et al., 2010).

Some factors should be considered in order to optimize *in vitro* seed germination process and the subsequent seedling development, among these, plant growth regulator types and concentrations, culture medium nutrient composition, photoperiod, post-sowing time (Roy and Banerjee, 2001).

Other important factors to take into account in the *in vitro* methodology are the pre-germination treatments with the major purpose of promoting seed priming and increase the physiological potential of seeds, uniformizing its vigor. Several chemical strategies are available for *in vitro* seed germination in *Capsicum* and others different plant species (Bello-Bello et al., 2010; Bora et al., 2014) and the influence of genotypes have been demonstrated (Shahzad and Sahai, 2014).

To determine the critical combination of factors that permit the improvement of *in vitro* germination in *Capsicum* seeds is an important strategy to develop multivariate protocols. Thus, this study aimed to evaluate the main effects of four factors potentially involved in the enhancement of *in vitro* seed germination in two genotypes of *C. baccatum*, the solution type for osmotic conditioning, germination medium, post-sowing time and genotypes, as well as the interactions between them on germination and cotyledon emerging rates, as a first step for establishing a cell suspension culture to produce secondary metabolites with commercial value in this species, besides demonstrate the potential of "two-level" full factorial experimental design for systemic establishing of the *in vitro* seed germination protocols.

2. Materials and Methods

2.1 Plant Material

The experiments were carried out in the Laboratório de Biotecnologia Vegetal, Departamento de Biotecnologia, UNESP – FCL, Assis-Brasil. Seeds of two *Capsicum baccatum* L. (pepper) genotypes were used, Pitanga (GN1) and Cambuci (GN2), obtained in street markets from Assis, São Paulo State (-22º39'42''S, -50º24'44''W). (.Seeds collected in matured fruits for both genotypes). Seeds were separated in two plots (200 seeds each) and used for assessing *in vitro* germination.

2.2 Seeds Pretreatment and Disinfestation

Seeds of both genotypes were submitted to pretreatment with 1% KNO_3 aqueous solution (w/v) or distilled water (control group) for 10 minutes in order to evaluate its influence on osmotic conditioning (pre soaking) and after were washed under running water and transferred to sterile environment. Pretreated seeds were surface disinfested in a laminar flow hood by soaking in 70% (v/v) ethanol for 2 minutes, rinsed with sterile distilled water, immersed in 1% (w/v) sodium hypochlorite (commercial solution) for 20 minutes and rinsed three times with sterile distilled water. After disinfestation, the seeds were kept submerged in sterilized distilled water until inoculation.

2.3 Sowing and Culture Medium

Disinfested seeds were aseptically inoculated in two different culture medium, M1, containing distilled water, 0.6% agar and 1.88 mg L^{-1} gibberellic acid (GA_3) ethanolic solution (the best concentration in previous experiments), and M2 (control), constituted of distilled water and 0.6% agar. For each culture medium were used 10 flasks of 500 mL containing 50 mL of medium (10 seeds /flasks), previously autoclaved at 121ºC, 1 atm for 15 minutes. Of the seeds pretreated with KNO_3 aqueous solution, 50 seeds were inoculated in M1 and 50 seeds in M2 and the same procedure was follow to the seeds pretreated with distilled water (control). All cultures were incubated at 25±2°C and 16 hours photoperiod.

2.4 Determination of Germination and Cotyledon Emerging Rates

Seeds were considered germinated when the radicle was 1 mm or greater. Germination rate was observed at 15 and 30 days and was calculated as the ratio between the number of seeds with radicle protrusion and the total number of seeds in each flask. The corresponding equation(1) used for germination rate was:

(1) $Y_{g_i} = N_{gp_i}/n$

Where:

Y_{g_i}: Germination rate in each flask (*i*=1, 2, 3, 4, 5); N_{gp_i}: number of seeds with radicle protrusion in each flask; n: total number of seeds in each flask (*n*=10).

Cotyledon emerging rate was also observed at 15 and 30 days, and it was determined as the ratio between the number of plants with cotyledon emerging and the total number of seeds in each flask. The mathematical expression (2) for calculate cotyledon emerging rate was:

(2) $Y_{c_i} = N_{cp_i}/n$

Where:

Y_{c_i}: cotyledon emerging rate in each flask (*i*=1, 2, 3, 4, 5); N_{cp_i}: number of plants with cotyledon emerging in each flask; n: total number of seeds in each flask (*n*=10).

2.5 Experimental Design

In order to define the main effects of each factor, osmotic conditioning (pre soaking treatment, gibberellic acid presence in germination culture medium, post-sowing time (15 and 30 days) and *Capsicum baccatum* L. genotypes (GN1 and GN2), as well as the interactions between factors on parameters germination and cotyledon emerging rates, a two-level full factorial design (2^4 experimental runs, five repetitions) was performed (Table 1). The level of significance (α) for statistical decisions was 0.05. Experimental planning and data analysis were carried out in Design-Expert 6.0 Software (Stat-Ease, Inc.; Minneapolis, MN, USA).

Table 1. Factors as well as their classification and levels considered in full two-level factorial (2^4) design to describe germination and cotyledon emerging rates associated to pepper seeds considered in the present study.

Factor	Type	Range	
		Low	High
Genotype (A)	Categorical	GN1	GN2
Osmotic conditioning solution (B)	Categorical	Water	1% KNO_3 w/v
Germination culture medium (C)	Categorical	Agar	Agar + GA_3
Post-sowing time (days) (D)	Numerical	15	30

3. Results

Primary results related to a two-level full factorial design ($2^4 = 16$) and five repetitions of each experimental combination are presented in Table 2. The combination of distilled water (osmotic conditioning solution) + agar (germination culture medium) was the most efficient procedure for germination rate in genotype Pitanga (GN1) and this finding was independent of the total time of germination, reaching the maximum level of 74% efficiency. For genotype Cambuci (GN2), the best combination was distilled water + agar-GA_3 for initial germination (15 days) and after this period (30 days) it was irrelevant to the seedling development, with values ranging between 76 and 86% germination efficiency. For the seedlings development, the 1% KNO_3 (w/v) + agar-GA_3 combination showed greater efficiency in cotyledon emergence for GN1 to 30 days, reaching a maximum of 56% efficiency. For GN2 the highest cotyledon emergence efficiency was confirmed in GA_3 presence regardless the association with pretreatment.

Table 2. Primary data corresponding to a two-level full factorial design ($2^4 = 16$) performed with five repetitions of each experimental combination. Germination parameters are represented as mean ± standard deviation (n=5).

Run	Genotype (A)	Osmotic conditioning solution (B)	Germination culture medium (C)	Time Days (D)	Y_G	Y_C
1	GN1	H_2O	Agar	15	0.56±0.21	0.04±0.09
2				30	0.74±0.11	0.18±0.08
3			Agar-GA_3	15	0.18±0.13	0.00±0.00
4				30	0.34±0.05	0.10±0.07
5		KNO_3 1% (w/v)	Agar	15	0.45±0.35	0.05±0.06
6				30	0.53±0.38	0.20±0.18
7			Agar-GA_3	15	0.48±0.22	0.16±0.11
8				30	0.72±0.24	0.56±0.17
9	GN2	H_2O	Agar	15	0.66±0.11	0.30±0.28
10				30	0.76±0.05	0.32±0.31
11			Agar-GA_3	15	0.75±0.10	0.45±0.13
12				30	0.83±0.05	0.58±0.13
13		KNO_3 1% (w/v)	Agar	15	0.58±0.08	0.20±0.19
14				30	0.84±0.11	0.22±0.18
15			Agar-GA_3	15	0.70±0.14	0.36±0.09
16				30	0.86±0.05	0.48±0.04

3.1 Germination Emerging

According to raw experimental data and confirmed by suitable statistical analysis, GN2 showed faster germination compared to GN1 (Table 3, Equation 3 and Figure 1a). The significant and positive term A (genotype) validate this finding (Table 3, Equation 3).

Table 3. Analysis of variance for selected factorial models, which describe the cotyledon emerging rates and any detail related to in vitro germination emerging.

Source	*In vitro* germination parameter under consideration	
	Germination rate	Cotyledon rate
	Probability values	
Model	< 0.0001*	< 0.0001*
A	< 0.0001*	< 0.0001*
B	0.3039	0.4082
C	0.4422	0.0001*
D	< 0.0001*	0.0003*
AB	0.2606	0.0009*
AC	0.0086*	0.1236
AD	0.7806	0.0708
BC	0.0014*	0.0327*
BD	0.4231	0.2566
CD	0.9869	0.1809
ABC	0.0011*	0.0416*
ABD	0.4231	0.2426
ACD	0.4231	0.9449
BCD	0.7806	0.3705
ABCD	0.4422	0.3525
Lack of fit	0.3448	0.1762

*This symbol represents significant tests (model goodness of fit and lack of fit) or model terms. A, B, C and D represent variables: genotype, osmotic conditioning solution, germination culture medium and time, respectively.

However, the influence of germination culture medium was different for both genotypes under study. The supplementation of agar basal medium with gibberellic acid (GA_3) was only effective for GN2 and the corresponding seeds demonstrated superior germination rate (Figure 1a). A contrary effect for GN1 was observed. The post-sowing time (D) showed positive significant effect, for this reason, 30 days are necessary in order to increase germination rate *in vitro* for both genotypes (Table 3, Equation 3).

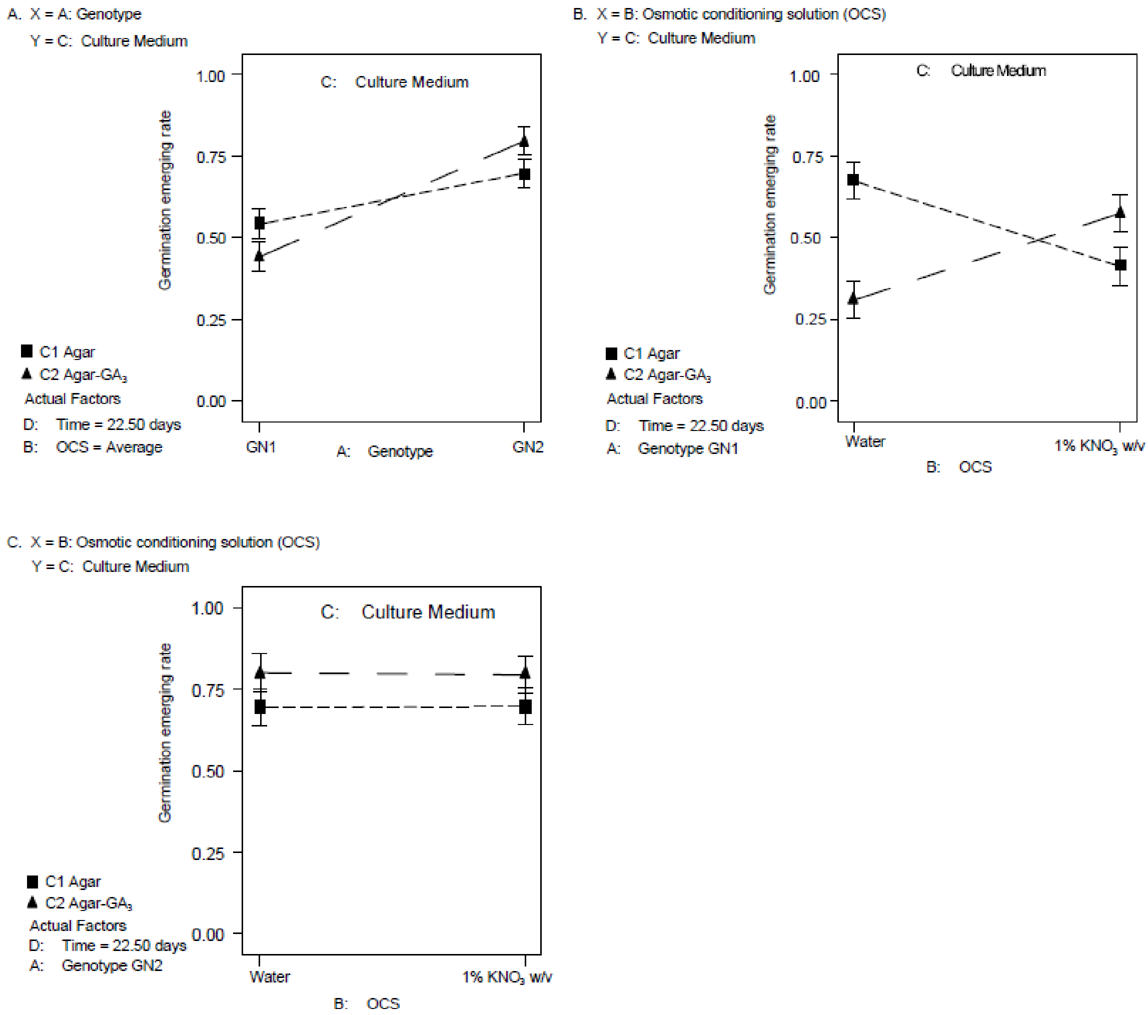
(3) $Y_G = 0.620 + 0.130 \cdot A + 0.082 \cdot D + 0.050 \cdot A \cdot C + 0.065 \cdot B \cdot C - 0.067 \cdot A \cdot B \cdot C$

Where:

Y_G: Yield of any detail related to germination emerging; A: Genotype; B: Osmotic conditioning solution; C: Germination culture medium; D: post-sowing time (days).

The water utilization as solution able to start and support seed germination (germination stimulating factor) demonstrated real positive effect only for GN1 (Figure 1b and 1c). For GN2, the impact of distilled water or 1% KNO_3 w/v, as solutions capable to start and support seed germination, was the same on germination rate (Figure 1c).

Fig. 1 Interaction graphs for in vitro germination rate. A: genotype-culture medium associated to yield of any detail related to germination emerging. B: osmotic conditioning solution (OCS)-culture medium associated to yield of any detail related to germination emerging for GN1. C: osmotic conditioning solution (OCS)-culture medium associated to yield of any detail related to germination emerging for GN2.



For GN1, the positive influence of distilled water in the induction of *in vitro* germination was associated to the germination culture medium, when utilized the basal medium composed only of agar, the best germination rate was observed. Contrary effect was confirmed for 1% KNO_3 solution, its best performance was in association to agar supplemented with GA_3 (Figure 1 b). This finding is also demonstrated for the significant interactions influence of $(B \cdot C)$ and $(A \cdot B \cdot C)$ in statistical model for germination rate (Table 3, Equation 3).

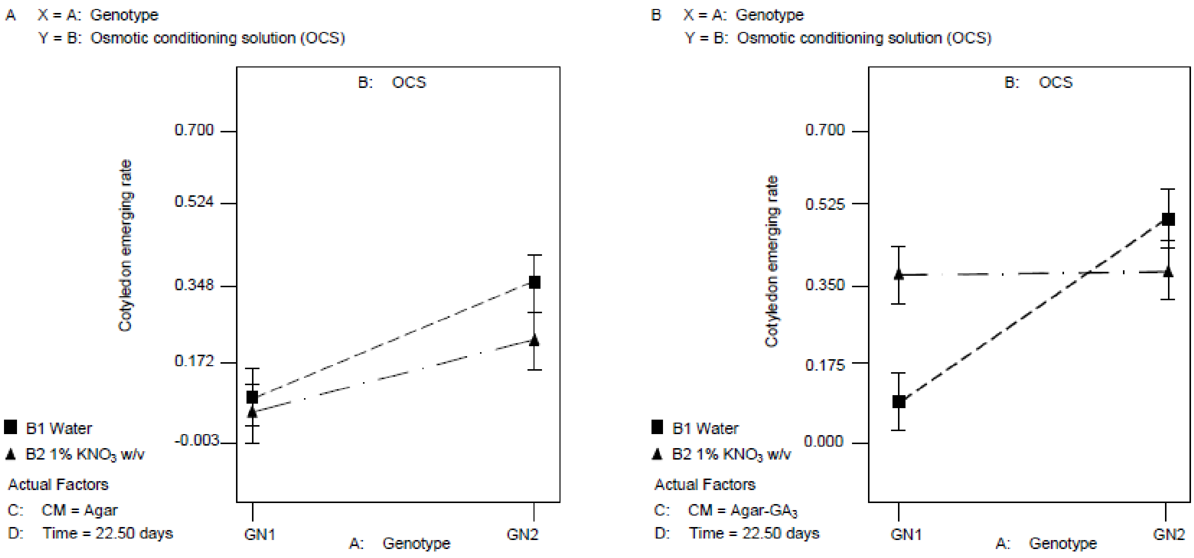
3.2 Cotyledon Emerging

Statistical modeling was also performed to describe cotyledon emerging (Equation 4, Table 3). From equation 4, it’s possible to conclude that genotype (A), germination culture medium (C) and post-sowing time (D) as well as binary interactions between $(A \cdot B)$ and $(B \cdot C)$ had significant influence on *in vitro* cotyledon emerging rate. As observed in germination, cotyledon emerging rate was higher at 30 days of the post-sowing time than to 15 days. The *in vitro* germination conditions were not enough to reduce the time to cotyledon emerging. The influence of osmotic conditioning solution (B) was confirmed in combination of genotype (A) and germination culture medium (C).

$$(4)\ Y_C = 0.260 + 0.100 \cdot A + 0.072 \cdot C + 0.068 \cdot D - 0.061 \cdot A \cdot B + 0.041 \cdot B \cdot C - 0.038 \cdot A \cdot B \cdot C$$

The choice of osmotic conditioning solution (distilled water or 1% KNO_3 w/v) was indifferent for cotyledon emerging rate in GN1 when water + agar was utilized as germination culture medium. On the other hand, this *in vitro* germination parameter was improved by distilled water compared to 1% KNO_3 w/v (higher than two-fold) for seeds corresponding to GN2 in the same culture medium (Figure 2a). Contrary findings were confirmed in culture medium supplemented with GA_3 (Figure 2b). In this case, the GN2 cotyledon emerging rate was not influenced by osmotic conditioning solution and GN1 showed higher cotyledon emerging rate when 1% KNO_3 w/v was used as osmotic conditioning solution (higher than two-fold, using distilled water as a reference).

Fig. 2 Interaction graphs for *in vitro* cotyledon emerging rate. A: genotype-osmotic conditioning solution (OCS) associated to yield of cotyledon emerging for agar as germination culture medium. B: genotype-osmotic conditioning solution (OCS) associated to yield of cotyledon emerging for agar-GA_3 as germination culture medium.



4. Discussion

The natural process of seed germination reflects a physiological sequence of events that is strongly influenced by external and internal factors, such as environmental conditions, dormancy, germination inhibitors or inducers. Such factors may act alone or integrated in order to transform the germination process in a biological event that determines the retake of metabolic activity and embryo plant growth with consequent emergence and development, radicle, hypocotyl and cotyledons (Bewley et al., 2013).

The parameters that determine the germination process and seedling development to establish the *in vitro* culture conditions, should follow the natural process sequence. Therefore, it is necessary to seek a balance between physiological seed quality, utilized genotype, the culture medium composition and the *in vitro* development itself. This study aimed to evaluate in an integrative approach the germinability of two *Capsicum baccatum* genotypes under variations in seed priming and composition of the culture medium.

Our results show that genotypes Pitanga (GN1) and Cambuci (GN2) from *Capsicum baccatum* had quiescent seeds in suitable stage of physiological maturation and vigor and that germinated in a relative short period (averaging 22 days, data not shown), producing healthy seedlings able to be use for explants extraction necessary for initiation of the *in vitro* callus culture. Such inference is based on Baskin and Baskin (2004) who stated a quiescent seed is capable of germinating in its fullest practicable extent for the physical environment factors, considering the imposed limits by their genotype.

In general, the germination period can be long and variable between different pepper species and the process could be irregular. The optimum germination temperature is about 30ºC and no specific light condition seems necessary for germination. However, several pretreatments can be used to enhance seed germination and seedlings emergence as e.g. potassium nitrate (KNO_3), gibberellic acid (GA_3) and sodium hypochlorite(Bosland and Votava, 2012; Cano-Vázquez et al., 2015; Cortez-Baheza et al., 2011).

Ex vitro pre-germination treatments has been used on different plant seeds species in order to increase the seeds germination, reducing the time between sowing and seedling emergence and enhance the seed tolerance to adverse environmental conditions. In this context, the priming is a promising treatment and it involves soaking seeds in natural or synthetic solutions under controlled conditions (temperature, light and time) followed or not by drying the seeds to the initial conditions of moisture content. The seed hydration to a certain level, allows the initial germination stages of the process to happen, however, without radicle protrusion. This process tends to result in homogeneous and quicker germination (Ibrahim, 2016; Manonmani et al., 2014; Paparella et al., 2015; Singh et al., 2015).

In *Capsicum annuum*, Kikuti et al. (2005) showed that conditioning with KNO_3 is effective to improve seed performance and the increase of exposure time to the priming solutions favors the process of *ex vitro* germination of sweet pepper. Batista et al. (2015) demonstrated that priming for the *ex vitro* germination of *Capsicum frutescens* is active in the emergence speed and the KNO_3 usage increases the seedlings dry weight, however the seeds quality is related to the germination treatments results.

Under the *in vitro* evaluated conditions, the genotype Pitanga (GN1) germinated better once priming with distilled water while genotype Cambuci (GN2) showed no difference in response to the pre-treatment with distilled water and KNO_3. For GN1 the KNO_3 did not influence the germination, water was enough to start the seeds germination. In contrast, GN2 showed indifferent behavior to priming treatments. Such behavior reflects the fact that each genotype has its own needs to start the germination process, determined by the conditions of seed formation and genetic composition, reflecting therefore divergence in the germination response (Popinigis, 1985).

As to cotyledons emergence, the genotype, the germination culture medium and post-sowing time as well as their interactions were more effective at 30 days post-sowing time, though such conditions were not sufficient to reduce this time. For GN1 the use of distilled water or 1% KNO_3 w/v was indifferent for cotyledon emerging rate when agar was utilized as the germination culture medium, indicating that this genotype is more vigorous and their genetic composition allows its development without the direct action of inductors or development stimulators.

For the germination behavior in the culture medium, was observed that the gibberellic acid (GA_3) presence influenced differentially seed germination and seedling growth. For GN2 (Pitanga) the culture medium containing gibberellic acid was effective in seed germination, but such treatment had no influence for GN1 (Cambuci). According to Vipranarayana et al. (2012), GA_3 also works effectively on *in vitro* seed germination of *Pterocarpus santalinus*

(Fabaceae - medicinal plant), probably breaks the dormancy and stimulates seed germination via synthesis of α-amylase and other hydrolyses.

According to Kucera et al. (2005) the dormancy process and germination are complex and controlled by various plants hormones encoded by a large number of genes affected by both developmental and environmental factors. The hormonal interactions determine the trigger mechanisms or control these events. In this context, gibberellic acid (GA_3) plays a fundamental role in the release of dormancy, increasing the embryo growth potential and overcoming the mechanical restraint conferred by the seed-covering layers, by weakening of the tissues surrounding the radicle. Gibberellic acid promotes germination being required for embryo cell elongation, for overcoming coat restrictions to germination of non-dormant and dormant seeds, and for inducing endosperm weakening (Gupta and Chakrabarty, 2013).

In the present study we choose the combination of hydropriming (in water) and halopriming (in solution of inorganic salts, i.e. KNO_3) as indicated in studies on the *ex vitro* seed germination of several species including *Capsicum* and that have shown variable germination patterns and seedling emergence (Aloui et al., 2014; Batista et al., 2015; Cortez-Baheza et al., 2011; Maiti et al., 2013; Smith and Cobb, 1991). Among the most diverse treatments suggested in the literature (water, NaCl, $CaCl_2$, KCl, PEG, KNO_3, GA_3, e.g.) those involving potassium nitrate (KNO_3) and gibberellic acid (GA_3) have shown promising results (Bosland and Votava, 2012), because such compounds assist in expression of the physiological seeds potential (Nascimento, 2005).

Regeneration *in vitro* studies in *Capsicum* have mostly evaluated the behavior in commercial genotypes of *Capsicum annuum*, *Capsicum chinense* and *Capsicum frutescens*. These studies have resulted in different protocols and observed a significant diversity of morphogenetic responses dependent on genotype, the explant type and the culture medium conditions (Akram et al., 2011; Bora et al., 2014; Gogoi et al., 2014; Grozeva et al., 2009; Manzur et al., 2013; Orlinska and Nowaczyk, 2015; Raj et al., 2015; Valadez-Bustos et al., 2009; Verma et al., 2013).

Among the various types of explants for the initial establishment of *in vitro* cultures, the seeds have advantages such as rapid growth, rapid availability of material for subsequent cultivation stages. However, regard the *in vitro* germination involving *Capsicum* species, different protocols available have directed attention to the seeds surface sterilizing procedures and the composition of the culture medium for germination. Most common sterilizing processes are the application of 0.1% HgCl and 70% ethanol, followed by inoculation in Murashige and Skoog basal culture medium. Hormones are mostly used after seedling is developed also in MS basal culture medium, not intending to reduce growth time, but to induce multiple shoot bud, callus formation, plant regeneration, flower induction, fruit development and rooting. Considerations for seed viability in germination rate or even seeds pretreatment, which can trigger or enhance germination, were not still reported (Manzur et al., 2013;Verma et al., 2013; Bora et al., 2014; Orlinska and Nowaczyk, 2015).

In this study we investigated the germination phenomena using a system's approach, as a function of the four essential parameters on*in vitro* cultivation (genotype, seed pretreatment, germination medium and time) and was confirmed that the best responses for the two *Capsicum baccatum* L. genotypes under consideration were different in germination rate terms, water + agar for Pitanga (GN1), and water + agar-GA_3 for Cambuci (GN2). On the other hand, the seedling development was stimulated by the combination of 1% KNO_3 + agar-GA_3 for genotype Pitanga (GN1), whereas the same optimal combination for rate of germination events was observed for Cambuci (GN2). In general, for both genotypes, the post-sowing time was not reduced using the osmotic conditioning solutions and germination culture media assessed.

The use of inducing agents or enhancers such as water, KNO_3 and/or GA_3, can be very effective to raise the germination process and enhance embryo development in different *Capsicum baccatum* genotypes. However, these parameters should be considered in an integrated manner so that it is possible to enhance or maximize the germination rate and lead to obtaining aseptic healthy seedling that can be employed as the explant source for *in vitro* culture protocols.

We concluded that for genotypes Pitanga (GN1) and Cambuci (GN2) both *Capsicum baccatum* L. the optimal treatments combinations were water (osmotic conditioning solution) + agar (germination culture medium) and water + agar-GA_3, respectively; and that the two-level full factorial experimental design (2^4) was effective to show differential responses for genotypes front of four main factors involved at *in vitro* germination phenomenon. It was established the most suitable conditions for the early bioprocess stage for producing secondary metabolites from *in vitro* culture of this specie, using a system's approach.

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CAPÍTULO 2 – Systemic approach for callus culture stage optimization – Using *Capsicum baccatum* L.var. pendulum as example

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Abstract

The oxidative stress and inflammations are associated with many diseases such as diabetes, cancer, cardiovascular and neurodegenerative diseases including atherosclerosis causing concern worldwide. As an alternative, the plants can play a key roledue to their pharmacological properties against these disorders; these attributes are most of the times associated with the presence of polyphenolic compounds. In this context, peppers can be a suitable alternative to obtain these compounds due their antioxidant properties. However, the presence of secondary metabolites (SM) and their phytochemical concentrations are affected by several abiotic factors. These problems can be solved with in vitro alternative techniques like plant cell and tissue culture methodologies. Aiming to optimize systemically the callus culture stage from Capsicum baccatum to produce SMs, this work evaluated explant's origin (root, hypocotyl and cotyledon), plant growth regulator (PGR) type (2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP) and a combination of 2,4-D/BAP at five-to-one ratio) and concentration (0.023, 1.138, 2.253, 5.000 and 10.000 mgL^{-1}). The main responses evaluated and their values for the optimized conditions were callus mass (225.03 mg), antioxidant activity (35.95%), total phenols (11.48mg of GAE/g DE) and flavonoids (15.92 mg of RU/g DE) production. These values were found with root explant using BAP at 1.138 mgL^{-1}. Thus, the multilevel factorial design was useful to find the better combination to achieve simultaneously highest values in callus mass, antioxidant activity and secondary metabolites, as well as to understand the relationships among factors and response variables in callus culture stage.

Keywords: *antioxidant activity; bioprocess optimization; callus culture; plant cell culture, polyphenolic compounds.*

Introduction

The oxidative stress and inflammations are associated with many diseases such as diabetes, cancer, cardiovascular and neurodegenerative diseases including atherosclerosis causing concern worldwide.[1]Thus, the search for alternatives, to minimize or control these health disorders, has become necessary for improving life quality in modern societies. In that sense, the plants have played a key role due to their pharmacological properties against these disorders. These attributes are most of the times associated with the presence of polyphenolic compounds, among them, phenolic acids and flavonoids are highlighted.[2]

Specifically, flavonoids are a group of molecules that have a common three ring chemical structure (C6–C3–C6) generated bythe secondary metabolism in certain plant species. They performa wide range of functions such as UV-light protection, defense against phytopathogens and antioxidant activity.[3]

Peppers,as are well-known, have a wide variety of phytochemicals with antioxidant properties and can be a suitable alternative to obtain these chemical structures. Among the peppers, the *Capsicum* genus, which is native to the tropical zones of Central and South America, stands out. It comprises an enormous variety of species with several fruit's shapes, sizes and flavors, having significant economic and cultural values. *Capsicum baccatum* is one of five main domesticated pepper species, in which also include *Capsicum annuum, Capsicum chinense, Capsicum frutescens,* and *Capsicum pubescens.*[1,4]

As a rule, the presence of secondary metabolites (SM) and their phytochemical concentrations are affected by several abiotic factors such as drought, temperature, altitude, salinity, metal ions, light, UV radiation and seasonality.[5,6]Another drawback, in obtaining them directly from plants, is related to the low natural disposition of these compounds. However, these problems can be solved with *in vitro* alternative techniques like plant cell and tissue culture methodologies to increase or avoid the intrinsic variability on SMs concentration. These *in vitro* tools have found extensive applications, both in academic and industrial environments. Many classes of SMs are synthesized, accumulated and sometimes exuded using some culture systems such as callus culture and cell suspension culture.[7,8]

In vitro plant cell cultures have several advantages for producing SMs compared with chemical synthesis or cultivated plant, such as controlled conditions for bioactive metabolite production (avoiding or lowering abiotic factors variance) and uniform quality and continuous supply of products using high production cell lines. Specifically, in the callus culture is taken pieces of tissue cut from surface-sterilized plants called explants. They are placed on appropriate solid growth medium and callus tissue will appear on the 14-60 days period. This is the initial stage inbioprocess that use *in vitro* plant cell approaches at large-scale.However, *in vitro* plant cell cultures, is still under development and have some limitations including low yields, genomic instability and scale-up difficulties.[9–11]

The aim of this paper was to optimize systemically the callus culture stage as initial step for developing a plant cell bioprocess to produce SMs with applications in food or pharmaceutical industries. The factors considered in the systemic approach werethe plant part (explant's origin) as well as PGR type and concentration in culture medium for callus propagation. The goals in callus culture optimization were to increase yield of callus mass, polyphenolic secondary metabolites and antioxidant capacity in extracts from *Capsicum baccatum* L.var. pendulum cells.

Material and Methods

Plant material

The explants were obtained from *Capsicum baccatum* L. var. pendulum (Cambuci) seedlings *in vitro* germinated (thereby avoiding disinfestation procedures). The seeds were obtained in street markets from Assis, São Paulo State (-22º39’42’’S,-50º24’44’’W) (Seeds collected in matured fruits for both genotypes). The experiments were conducted at Laboratório de Biotecnologia Vegetal, UNESP – FCL, Assis/SP-Brasil.

Optimization of callus culture

The explants (in average 7 mg) were obtained from seedling (Pretreated seeds with 1% KNO_3 aqueous solution (w/v) for 10 minutes and *in vitro* germinated in culture medium containing distilled water, 0.6% agar and 1.88 mg L^{-1} gibberellic acid) with cotyledons appearance. The plant sections used to obtain callus were roots, hypocotyls and cotyledons. With the aid of sterilized tweezers and scissors, explants were cut and inoculated in Petri dishes (15x100 mm) containing 35 mL Murashige and Skoog culture medium (MS) (pH 5.75) solidified with Phytagel (Sigma Chemical Co., St. Louis, MO) 4gL^{-1} and supplemented with 3% sucrose and different PGRs with concentration according to the experimental design. The culture media were previously autoclaved at 121ºC, 1 atm during 20 minutes.

The three chosen variables and corresponding levels for understanding and setting the *Capsicum baccatum* L. var. pendulum callus culture cells were explant’s origin (root, hypocotyl and cotyledon), PGR (2,4-dichlorophenoxyacetic acid (2,4-D), benzylaminopurine (BAP) and a combination of 2,4-D/BAP at five-to-one ratio and concentration (0.023, 1.138, 2.253, 5.000 and 10.000 mgL^{-1}). A multilevel factorial design was performed in order to optimize and model statistically the influence of three mentioned input variables on total phenols, flavonoids and antioxidant activity from hydroalcoholic extracts as well as callus mass and budding derived from callus culture. The corresponding experimental runs or combinations are shownin Table 1.

All procedures were done in a sterile environment (vertical laminar flow hood). Each Petri dish had five explants and the experimental combinations were randomly performed in triplicate (48.89%), in duplicate (37.78%) and in single sample (13.33%). The cultures were kept at 30°C in the dark for 30 days in incubator chamber to callus growth (Eletrolab, model EL101/3, São Paulo, Brazil).

Extracts

Each Petri dish with viable calluses, they were frozen and lyophilized (LIOBRAS, model Loitop L101, São Carlos, Brazil) (24h-36h) to obtain the dry mass. Then it was macerated with $N_{2(l)}$(77 K) to obtain the vegetable powder. The solutions were prepared in the ratio of 5 mg (powder) per 1 mL in ethanol 70%(v/v). Then, they were put in ultrasound bath (seven minutesat 30 KHz), and after were centrifuged for 15 min at 2000 rpm(QUIMIS, micro processed centrifuge, model: Q222TM216, Diadema, Brazil). The supernatant was used for the chemical assays.

In vitro antioxidant activity

The *in vitro* antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) methodology described by Blois[12] with modifications. In each tube was added absolute ethanol (1.250 mL), acetate buffer (1.000 mL) (100 mM pH 5.5), ethanolic solution of DPPH (0.250mL) (500 μM) and the sample (0.050 mL). The control tube was done excluding the sample addiction. The reaction mixtures were stirred by vortex and kept in the dark for 30 min. The absorbance was measured using a Femto-600 Plus UV/vis spectrophotometer (São Paulo, Brazil) at 517 nm. The samples quantifications were done in triplicate to guarantee statistical robustness of the results and the absorbance values were expressed as percentage by the following equation (1):

$$\%AA = \left[\frac{(A_c - A_S)}{A_C}\right] \times 100 \quad (1)$$

Where: % AA is the antioxidant activity, A_C is the control absorbance and A_S is the sample absorbance.

Quantification of callus wet weight and budding index

The budding index (%) was determined to characterize the effectiveness of callus formation in each experimental condition, this parameter was defined considering explants number to callus number ratio. On the other hand, the wet weight of each callus was measured in analytical balance.

Total phenol and flavonoid assays

The content of total phenolic compounds was quantified using the Folin-Ciocalteu colorimetric method modified by Singleton and Rossi.[13] In a reaction mixture containing extract (0.050 mL) was added distilled water (2.500 mL) and Folin-Ciocalteu reagent (0.250 mL). After 3 minutes, saturated (25% w/v) Na_2CO_3 solution (0.700 mL) and distilled water (1.500 mL) were added. Then were stirred by vortex and kept standing for 60 min. The absorbance was measured using a Femto-600 Plus UV/vis spectrophotometer (Femto, São Paulo, Brazil) at 765 nm. The samples were run in triplicate for statistical analysis and the results expressed as milligram gallic acid equivalent (GAE) per gram of dry weight of extract (mg of GAE/g DE).

The total flavonoids content was determined by Vis spectrophotometry and the samples prepared based on flavonoids complexation with $AlCl_3$ as in colorimetric method described by Zhishen et. al.[14] In a reaction mixture containing extract (0.500 mL) was added ethanol 70% (2.000 mL) and 5% (w/v) $NaNO_2$ solution (0.250 mL). After 6 minutes, 10% (w/v) $AlCl_3/H_2O$ solution (0.250 mL), 1M NaOH solution (1.500 mL) and distilled water (0.500 mL) were added. Then the reaction mixtures were stirred by vortex and kept standing for 15 min. The absorbance was measured using a Femto-600 Plus UV/vis spectrophotometer at 510 nm. Thesampleswere also run in triplicateand the results expressed as the rutin equivalent per gram of dry weight of extract (mg of RU/g DE).

Statistical Analysis

Experimental planning and data analysis from multilevel factorial design were carried out in Design-Expert software (Trial version 10.0.3.1, Stat-Ease, MN, USA). The level of significance (α) for statistical decisions was 0.05. The optimization of callus culture variables for maximizing callus mass and antioxidant activity as well as polyphenolic compounds and flavonoids were done by means of Derringer and Suich's desirability function[15] implemented in the same software.

Results and Discussion

Current study was performed to establish the initial stage (callus culture) of a biotechnology to produce SM from *Capsicum baccatum* L. var. pendulum. The use of explants herein from seedling lied on the previous observations which confirmed that young tissue is more responsive to callus initiation than mature plant tissues.[16] Besides, drastic tissue decontamination procedures and time consuming and low controlled protocols, when explants are taken from species in natural environments, were avoided. Among the 45 treatments (15 for each explant's origin), 13 did not form callus (Electronic Supplementary Material 1). The failed experiments were distributed as follow: six in root explants, five in hypocotyl and two in cotyledon. The successful use of cotyledon in the present work as explant source for obtaining high budding indexes respect to other plant tissues utilized *in vitro* regeneration techniques has been also confirmed for other vegetable species, including Chili peppers.[17–19] This superiority may be related to development of meristematic zones in subepidermal cells on the adaxial side of cotyledon explant.[20] However, the hormone type and concentration can change this regularity.[19]

The response variables utilized for describing and optimizing the callus culture stage were analyzed individually (following sections). All models for them were significant and the statistical assessment for the studied factors was also performed. Table 2 shows the statistics obtained from Analysis of Variance (ANOVA; $p < 0.05$) in each response. The statistical models were explained and discussed using derived graphs from polynomial models comprising significant terms (Table 2). The callus phytochemical parameters were just considered in samples with sufficient dry mass.

Wet weight

The callus mass varied widely among the assessed experimental combinations. The best result for root was 498.23 mg using 2,4-D/BAP (5:1 ratio) at 1.138 mgL^{-1}. Similarly, the highest callus mass (862.43 mg)for hypocotyl was obtained utilizing the same hormone mixture and concentration. However, for cotyledon, the best value of callus weight (519.13 mg)was reached with 2,4-D at 2.253 $mg.L^{-1}$ (Electronic Supplementary Material 1).The Figure 1A shows the interaction among explant's origin and PGR or mixture; concentration factor was adjusted in the mean. It was observed 2,4-D had better response for cotyledon and similar response for root or hypocotyl. On the other hand, the effect of BAP utilization in culture medium was similar in the three evaluated explants. Nevertheless, the 2,4-D/BAP had showed better response for hypocotyls and similar response for root and cotyledon. Both hormones are quoted in callus formation due their capacity in promote cell division and growth, however, this higher response in calluses using 2,4-D/BAP can be related with the synergism between auxin-cytokinin interaction.[21,22]

The Figure 1B shows the interaction among explant's origin and PGR concentration; PGR factor was adjusted in the mean. It was confirmed superior performance in callus weight for hypocotyl and cotyledon explants when PGR concentration was in 1.138-2.253 mg L^{-1} range but, the 1.138 mgL^{-1} concentration was more suitable for root explant. On the other hand, the Figure 1C shows the interaction among PGR and PGR concentration; explant's origin factor was adjusted in the mean. The 1.138 mgL^{-1} concentration at was the optimum value when the PGR mixture 2,4-D/BAP (5:1 ratio) was included in culture medium. These differences in the callus mass obtained at different concentrations and explant's origin can be related to the different sensibility of explant's origin; for example, roots are more sensitive than hypocotyls in relation to auxins. Another point is that concentrations higher than the optimum are inhibitory. Besides, the quantity applied in the medium can influence the synthesis and degradation of endogenous hormones used in callus propagation.[22,23]

Budding ratio

The budding ratio (%) was evaluated to define its relationship with callus mass. It was confirmed a direct correlation between callus mass and budding ratio (Table 2). The highest callus weights for each explant's origin: 862.43 mg (hypocotyl); 519.13 mg (cotyledon), 498.23 mg (root), had 93.33%, 93.33% and 86.67% budding ratio, respectively.

The Figure 2 shows the interaction among explant's origin and PGR, concentration factor was adjusted in the mean. It was possible to observe the same hormone response in the use of BAP and 2,4-D/BAP for root, hypocotyl and cotyledon. The use of 2,4-D improved budding index in cotyledon respect to hypocotyl and root explants, meanwhile, it was observed a similar behavior of this callus culture parameter for the other PGR combinations under study for cotyledon. Thus, it was possible to confirm a great likeness between callus mass and budding index models (Figure 1A and Figure 2), and so the hormone type/mixture and concentration seem to influence like the processes of induction of callus from explants and stimulation of cell division.

The highest observed values of callus mass and budding ratio for a hypocotyl explant using 2,4-D/BAP is in harmony with results derived from previous study carried out with *Capsicum annuum* and the synergic effect of auxin and cytokinin hormones in callus culture.[19,22]

Antioxidant activity

The callus antioxidant activity (AA%) showed significantly differences in performance for each treatment. The ANOVA results showed no significant influence of explant’s origin (Table 2). The PGRs and their concentrations resulted different AA%. For root, the AA% varied from 1.14 to 41.48%. For hypocotyl, this response varied from 1.47 to 9.56%. On the other hand, in cotyledon, values of AA% were in 0.41-11.19% range.

BAP had better response in root and hypocotyl. However, there was no difference among the hormones for cotyledon (Figure 3A). On the other hand, the Figure 3B shows that the BAP use at 1.138 $mg.L^{-1}$ had an extremely better response. The best treatment (root using BAP at 1.138 mgL^{-1}), with 41.48%, has a higher AA% than a previous value that reported by Bertão et al.[24] in *C. baccatum* fruits – 22.98% at 5.0 mgL^{-1} extract concentration (the same used is this study) and 32.89% at 10.0 mgL^{-1} extract concentration. Thus, it is demonstrated the suitability of the callus culture for producing SMs with antioxidant activities in *C. baccatum*.

Total phenols(TP)

Total phenols varied widely (Electronic Supplementary Material 1). As observed in AA%, the ANOVA results (Table 2) showed no significant influence of explant's origin. Specifically, the TP values for root explant varied from 0.36 to 13.13 mg of GAE/g DE. For hypocotyls and cotyledons,values varied from 0.27 to 10.00 mg of GAE/g DE and from 0.15 to 6.49 mg of GAE/g DE, respectively.

The interaction graph among explant's origin and PGR or mixture (Figure 4A) revealed that BAP had better response in the TP amount, except for cotyledon. Besides, the Figure 4B shows the interaction among concentration factor and PGR. The TP amounts obtained in the best treatment (using BAP at 1.138 mgL^{-1}) were 13.13 mg of GAE/g DE for root, 10.00 mg of GAE/g DE for hypocotyls and 6.48 mg of GAE/g DE for cotyledon. The results follow the same pattern found to (AA%) suggesting a correlation between both responses that has been quoted in recent researches about higher AA% related with TP.[1,2]Krishnan et al.also reported for *Gynura procumbens* higher TP concentration in root callus than those values from other plant part callus (leaf with 0.483, stem with 0.559, and root with 0.891 mg of GAE/g Fresh Weight).[25]

Flavonoids

The flavonoids did not vary widely among the treatments, except for four treatments using BAP(Table 1, Electronic Supplementary Material 1).The treatments using 2,4-D and 2,4-D/BAP (5:1 ratio) showed flavonoids contents inferior to 5.0 (mg of RU/g DE). Conversely, BAP treatments varied from 8.89 to 20.44 (mg of RU/g DE).

The treatments with higher flavonoids content were similar to those with higher TP content and AA% (Figure 3, Figure 4, Figure 5), reasserting the relation of these compounds with antioxidant activity.[7] However, not all AA% can be related only with TP or flavonoids, but also to other non-phenolic compounds like terpenoids.[26]

Optimization

The optimization was done by desirability function, the factors under study were maximized and an importance scale among the responses was attributed: callus mass (5), AA% (4), TP (3) and flavonoids (3). The budding was adjusted to the range due its relation with callus mass. The best result was defined for root explant using BAP at 1.138mgL^{-1}. This factor set allows the best compromise among callus mass (225.03 mg), antioxidant activity (35.95%), total phenols (11.48 mg of GAE/g DE) and flavonoids (15.92 mg of RU/g DE) production. This result was obtained avoiding the several abiotic factors that may reduce their concentration and unable to be realized the large scale production.[5,6]

Conclusions

This work is likely the first study that uses a systematic approach in order to describe and optimize callus culture stage to initiate a long-term culture with potential pharmaceutical properties using a widely-consumed pepper (*Capsicum baccatum*). The multilevel multifactorial design was successful in found the better combination to higher values in callus mass, antioxidant activity and secondary metabolites. The flavonoid and total phenol content showed direct relation with the callus antioxidant properties.

The hypocotyl explant in combination with 2,4-D/BAP (5:1 ratio) at 1.138 mg L^{-1} showed the best callus mass values. However, the multiple optimization considering simultaneously callus mass, total phenols, flavonoids and antioxidant activity resulted in the following combination of factor: root explant, benzylaminopurine hormone at 1.138 mg L^{-1}.

Acknowledgments

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Tables

Table 1. Experimental runs (15 for each explant's origin) associated to multilevel factorial design for modelling and optimizing callus culture stage from *Capsicum baccatum* L. var. pendulum cells. All combinations were randomly performed in triplicate (48.89%), in duplicate (37.78%) or in single sample (13.33%).

Explant's origin	PGR or mixture	PGR Concentration (mg L^{-1})
Root, Hypocotyl or Cotyledon	2,4-D	0.023
		1.138
		2.253
		5.000
		10.000
	BAP	0.023
		1.138
		2.253
		5.000
		10.000
	2,4-D/ BAP (5:1 ratio)	0.023/0.0046
		1.138/0.2276
		2.253/0.7510
		5.000/1.0000
		10.000/2.0000

Table 2. Analysis of variance (ANOVA) for each response variable model. Probability values associated to the statistical significance tests for models, terms and model lack of fit of the coded response variables used to describe callus cultures stage are reported.

Source	Response variables				
	Callus Mass (mg)	Budding index (%)	Antioxidant Activity (%)	Total Phenols (mg of GAE/g DE)	Flavonoids (mg of RU/g DE)
Model significance	<0.0001	<0.0001	<0.0001	< 0.0001	<0.0001
Term significance					
A - Explant's origin	<0.0001	<0.0001	-	-	-
B- PGR or mixture	0.0010	-	< 0.0001	< 0.0001	< 0.0001
C-PGR Concentration	<0.0001	<0.0001	< 0.0001	< 0.0001	< 0.0001
AB	<0.0001	<0.0001	0.0351	0.0031	-
AC	0.0179	<0.0001	-	-	-
BC	<0.0001	<0.0001	< 0.0001	< 0.0001	< 0.0001
ABC	0.0058	0,0005	-	-	-
Lack of fit test	-	-	0.9362	0.9552	0.8695

The symbol (-) represents the no significant statistical tests.

Caption to Figures

Figure 1. Interaction graphs for adjusted model associated to callus mass. A: Interaction between explant's origin and PGR type or mixture. B: Interaction between explant's origin and PGR concentration. C: Interaction between PGR type or mixture and PGR concentration.

Figure 2. Interaction graph between explant's origin and PGR type or mixture for adjusted model associated to budding index.

Figure 3. Interaction graphs for adjusted model associated to antioxidant activity. A: Interaction between explant's origin and PGR type or mixture. B: Interaction between PGR type or mixture and PGR concentration.

Figure 4. Interaction graphs for adjusted model associated to total phenols. A: Interaction between explant's origin and PGR type or mixture. B: Interaction between concentration factor and PGR type or mixture.

Figure 5. Interaction graph between concentration factor and PGR type or mixture for adjusted model associated to flavonoids.

Figure 1A

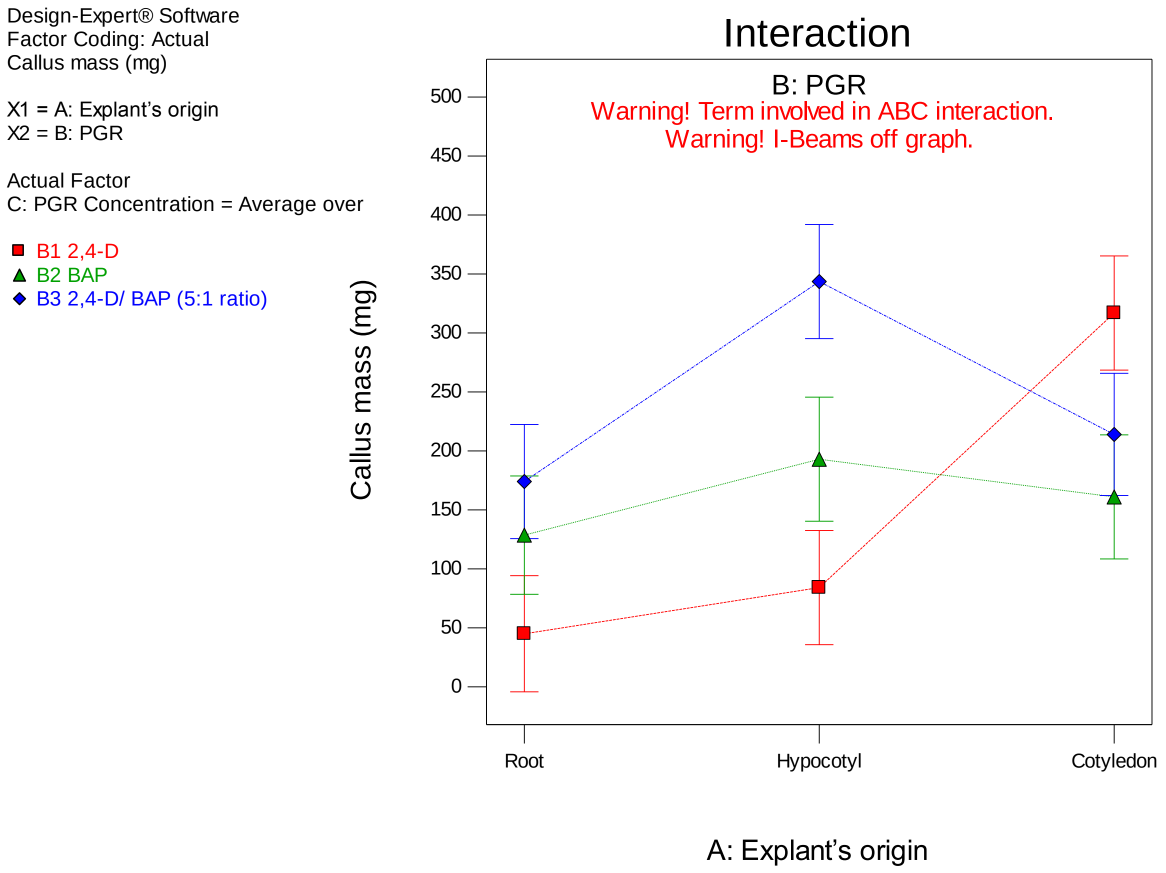

Design-Expert® Software
Factor Coding: Actual
Callus mass (mg)
X1 = A: Explant's origin
X2 = B: PGR
Actual Factor
C: PGR Concentration = Average over
B1 2,4-D
B2 BAP
B3 2,4-D/ BAP (5:1 ratio)
Interaction
B: PGR
Warning! Term involved in ABC interaction.
Warning! I-Beams off graph.
Callus mass (mg)
500
450
400
350
300
250
200
150
100
50
0
Root
Hypocotyl
Cotyledon
A: Explant's origin

Figure 1B

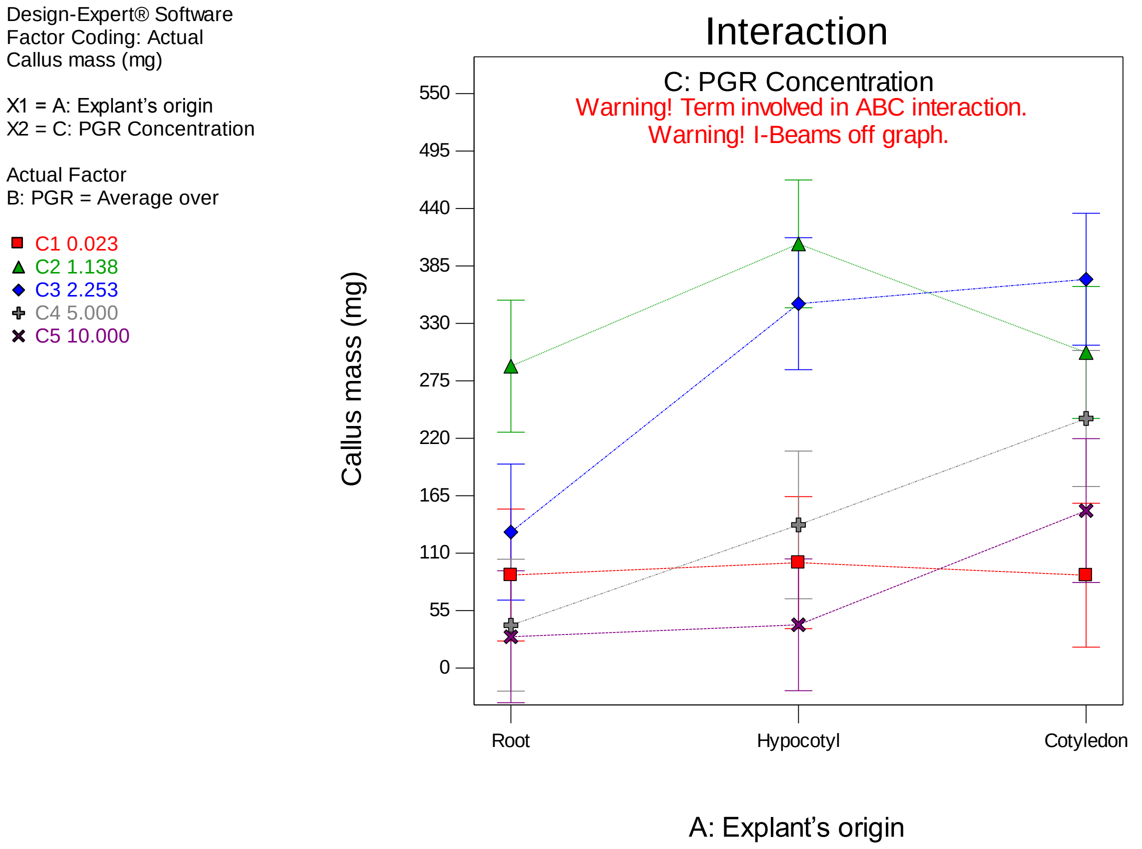

Design-Expert® Software
Factor Coding: Actual
Callus mass (mg)
X1 = A: Explant's origin
X2 = C: PGR Concentration
Actual Factor
B: PGR = Average over
C1 0.023
C2 1.138
C3 2.253
C4 5.000
C5 10.000
Interaction
C: PGR Concentration
Warning! Term involved in ABC interaction.
Warning! I-Beams off graph.
Callus mass (mg)
550
495
440
385
330
275
220
165
110
55
0
Root
Hypocotyl
Cotyledon
A: Explant's origin

Figure 1C

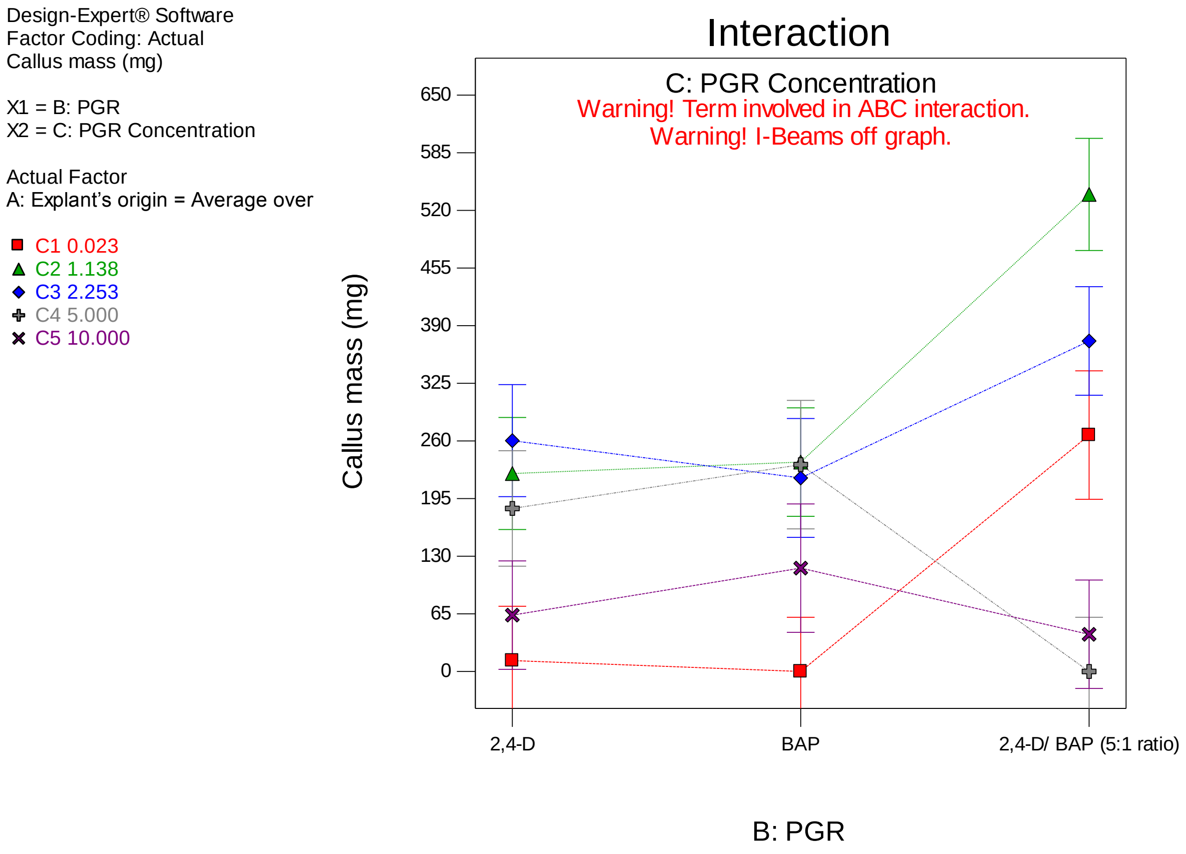

Design-Expert® Software
Factor Coding: Actual
Callus mass (mg)
X1 = B: PGR
X2 = C: PGR Concentration
Actual Factor
A: Explant's origin = Average over
C1 0.023
C2 1.138
C3 2.253
C4 5.000
C5 10.000
Interaction
C: PGR Concentration
Warning! Term involved in ABC interaction.
Warning! I-Beams off graph.
Callus mass (mg)
650
585
520
455
390
325
260
195
130
65
0
2,4-D
BAP
2,4-D/ BAP (5:1 ratio)
B: PGR

Figure 2

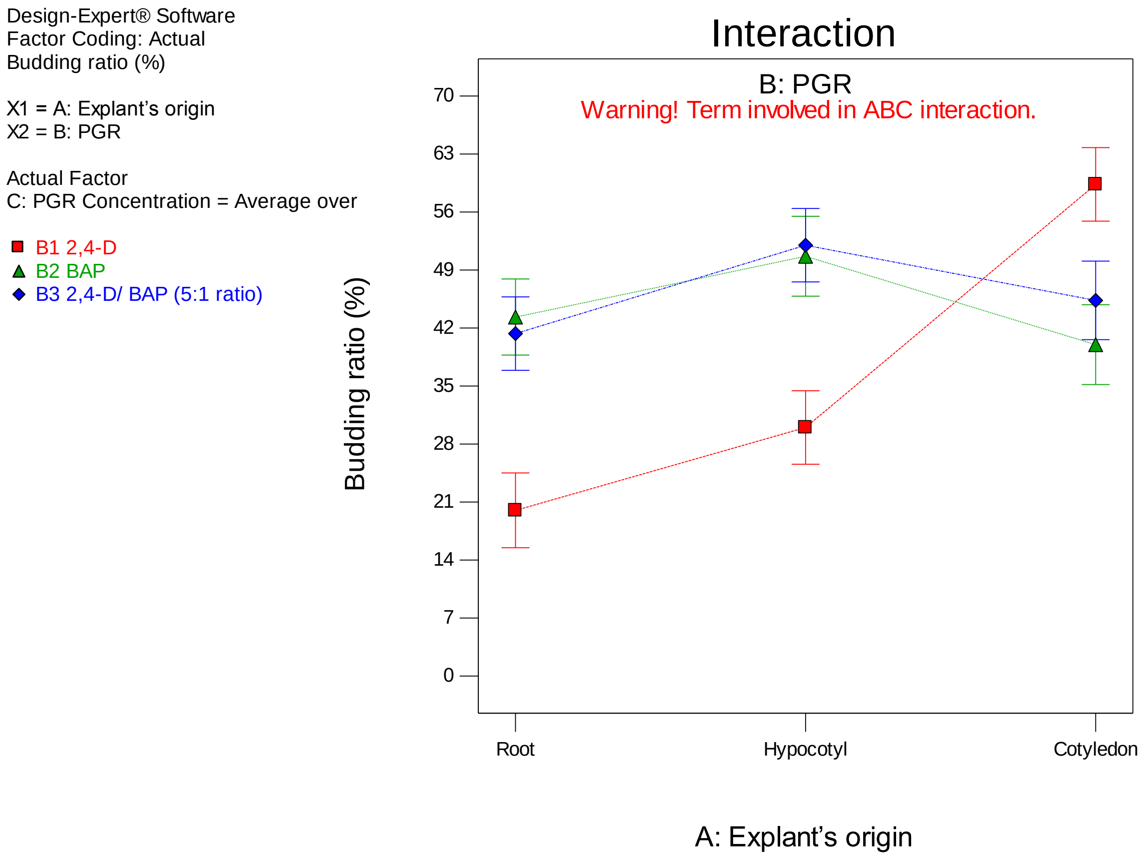

Design-Expert® Software
Factor Coding: Actual
Budding ratio (%)
X1 = A: Explant’s origin
X2 = B: PGR
Actual Factor
C: PGR Concentration = Average over
B1 2,4-D
B2 BAP
B3 2,4-D/ BAP (5:1 ratio)
Interaction
B: PGR
Warning! Term involved in ABC interaction.
Budding ratio (%)
70
63
56
49
42
35
28
21
14
7
0
Root
Hypocotyl
Cotyledon
A: Explant’s origin

Figure 3A

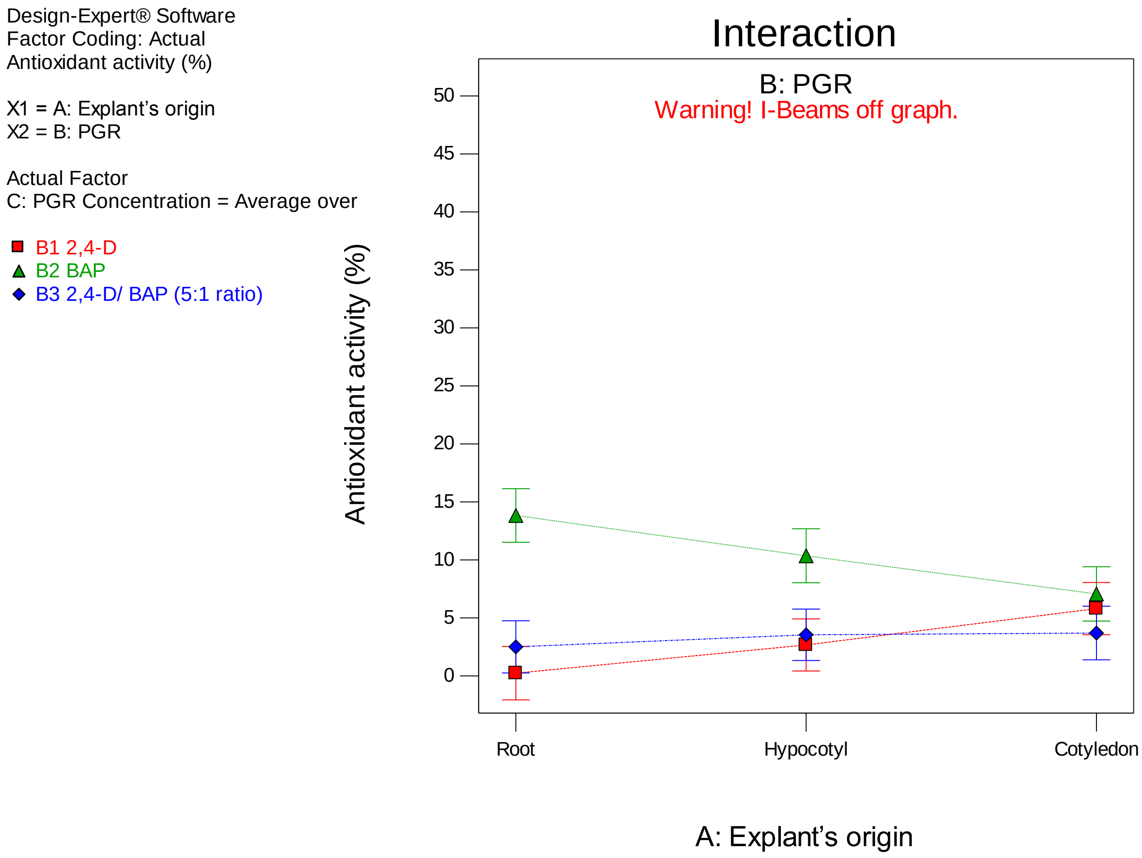

Design-Expert® Software
Factor Coding: Actual
Antioxidant activity (%)
X1 = A: Explant's origin
X2 = B: PGR
Actual Factor
C: PGR Concentration = Average over
B1 2,4-D
B2 BAP
B3 2,4-D/ BAP (5:1 ratio)
Interaction
B: PGR
Warning! I-Beams off graph.
Antioxidant activity (%)
50
45
40
35
30
25
20
15
10
5
0
Root
Hypocotyl
Cotyledon
A: Explant's origin

Figure 3B

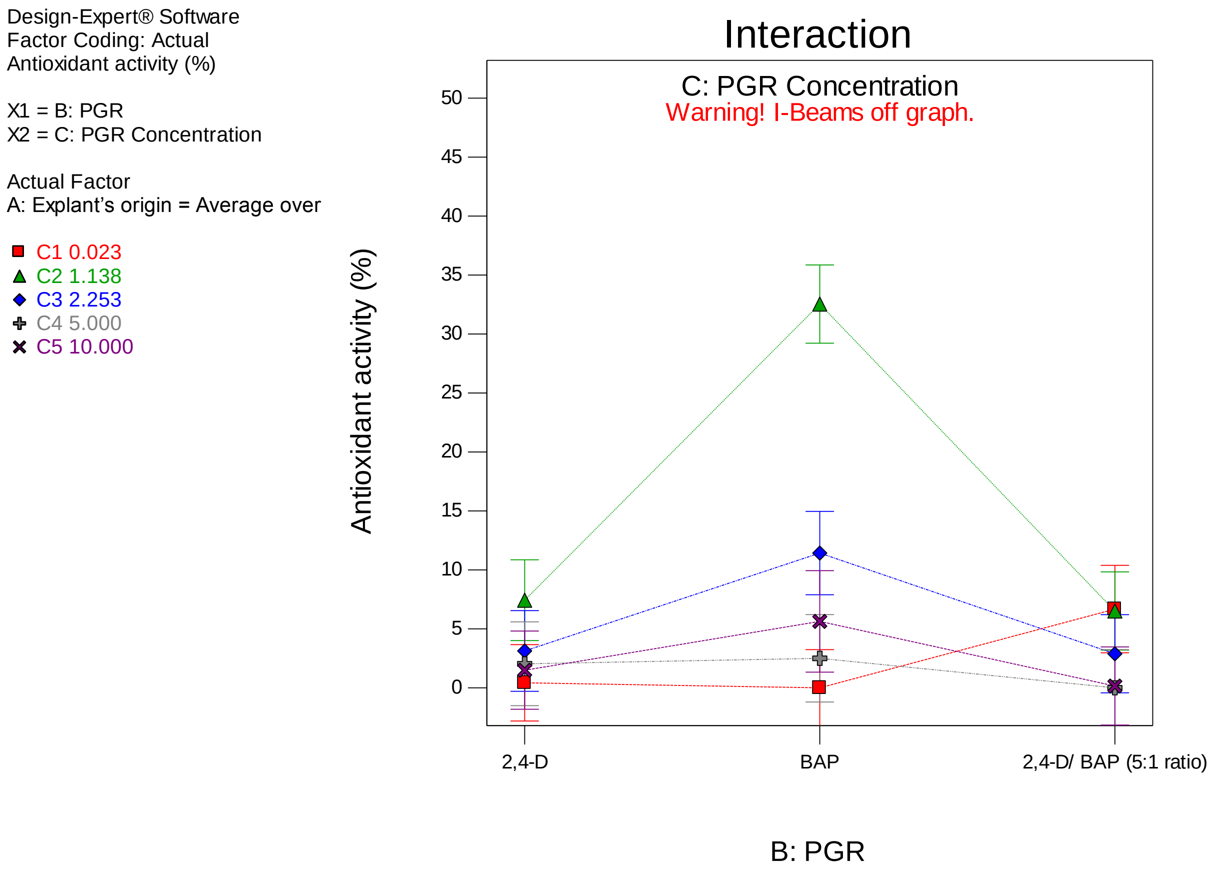

Design-Expert® Software
Factor Coding: Actual
Antioxidant activity (%)
X1 = B: PGR
X2 = C: PGR Concentration
Actual Factor
A: Explant's origin = Average over
C1 0.023
C2 1.138
C3 2.253
C4 5.000
C5 10.000
Interaction
C: PGR Concentration
Warning! I-Beams off graph.
Antioxidant activity (%)
0
5
10
15
20
25
30
35
40
45
50
2,4-D
BAP
2,4-D/ BAP (5:1 ratio)
B: PGR

Figure 4A

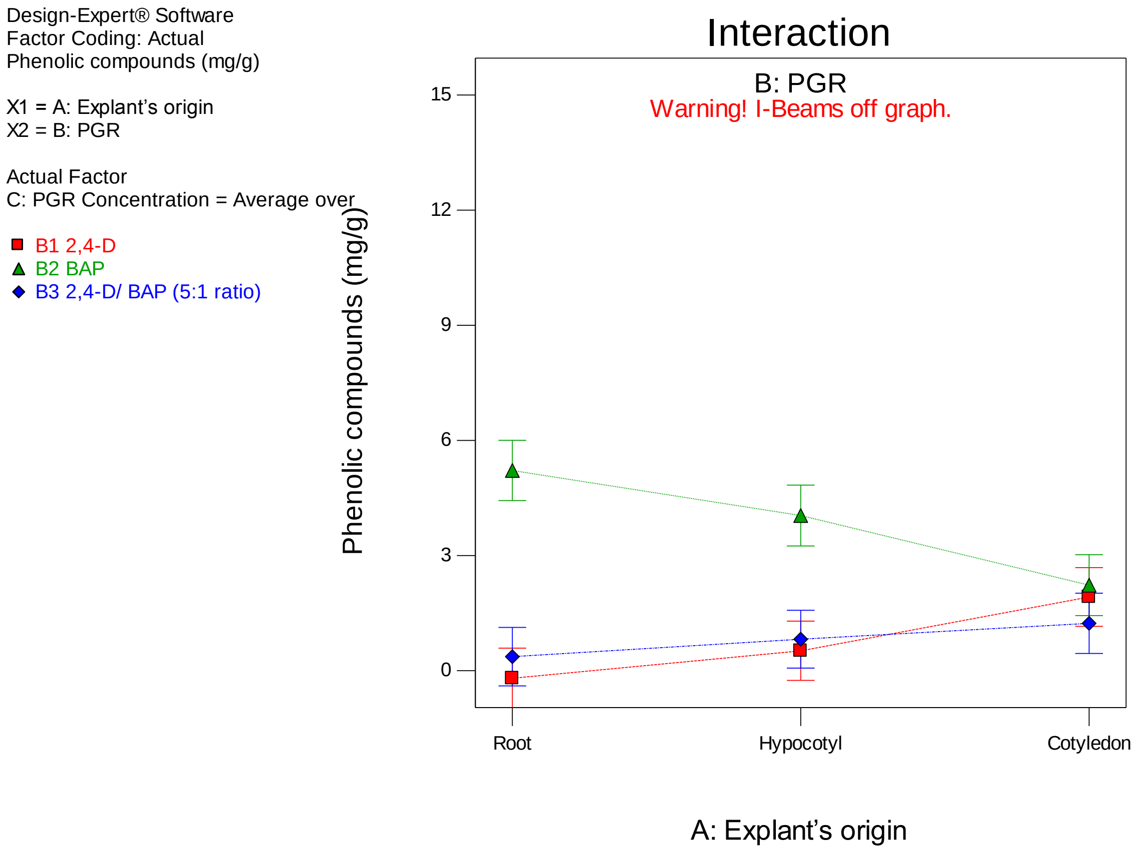

Design-Expert® Software
Factor Coding: Actual
Phenolic compounds (mg/g)
X1 = A: Explant's origin
X2 = B: PGR
Actual Factor
C: PGR Concentration = Average over
B1 2,4-D
B2 BAP
B3 2,4-D/ BAP (5:1 ratio)
Interaction
B: PGR
Warning! I-Beams off graph.
Phenolic compounds (mg/g)
15
12
9
6
3
0
Root
Hypocotyl
Cotyledon
A: Explant's origin

Figure 4B

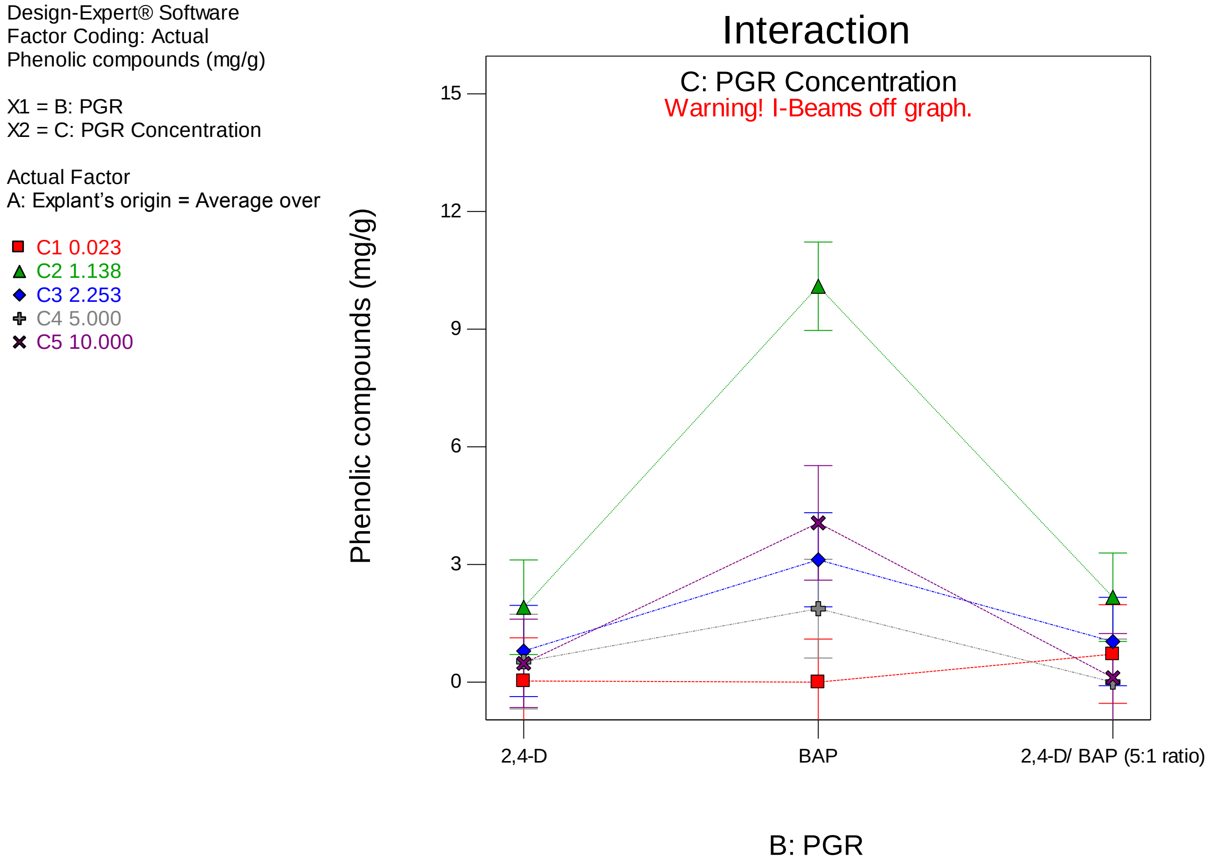
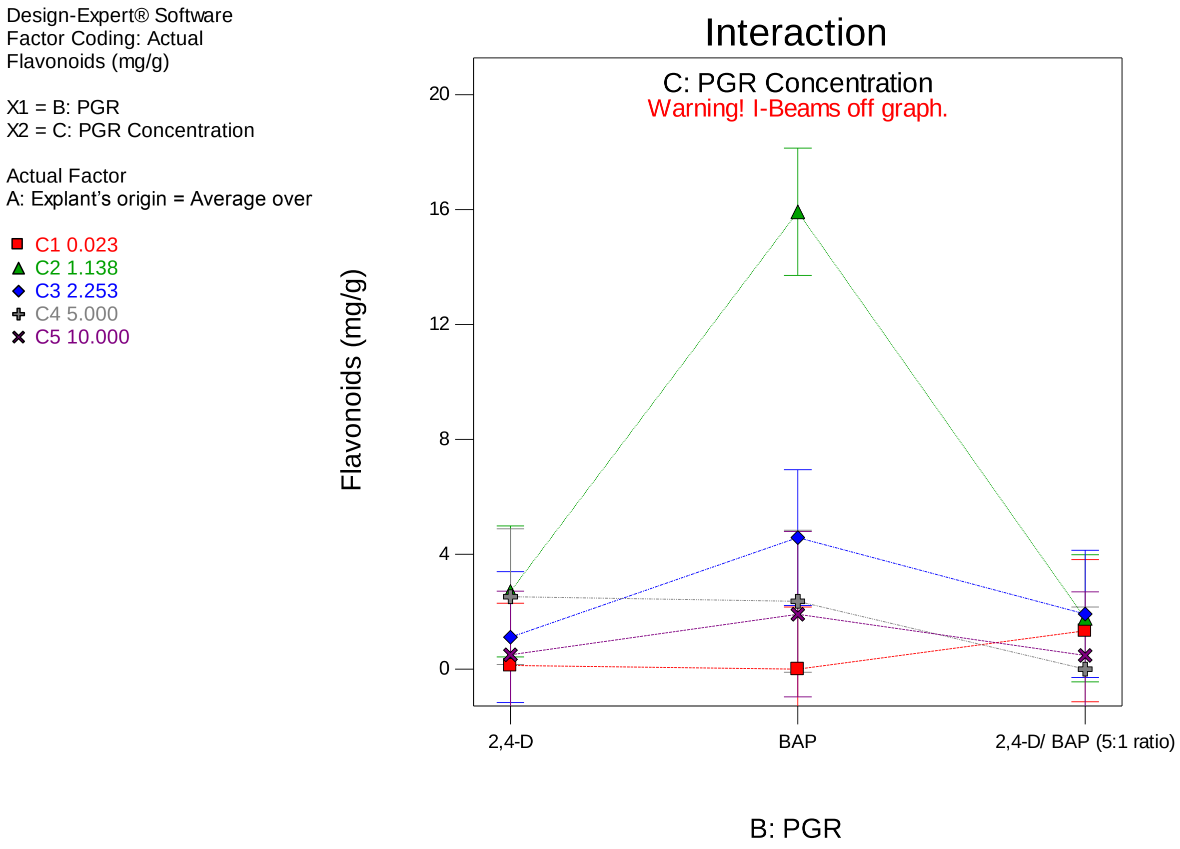

Design-Expert® Software
Factor Coding: Actual
Phenolic compounds (mg/g)
X1 = B: PGR
X2 = C: PGR Concentration
Actual Factor
A: Explant's origin = Average over
C1 0.023
C2 1.138
C3 2.253
C4 5.000
C5 10.000
Interaction
C: PGR Concentration
Warning! I-Beams off graph.
Phenolic compounds (mg/g)
15
12
9
6
3
0
2,4-D
BAP
2,4-D/ BAP (5:1 ratio)
B: PGR

Figure 5


Design-Expert® Software
Factor Coding: Actual
Flavonoids (mg/g)
X1 = B: PGR
X2 = C: PGR Concentration
Actual Factor
A: Explant's origin = Average over
C1 0.023
C2 1.138
C3 2.253
C4 5.000
C5 10.000
Interaction
C: PGR Concentration
Warning! I-Beams off graph.
Flavonoids (mg/g)
20
16
12
8
4
0
2,4-D
BAP
2,4-D/ BAP (5:1 ratio)
B: PGR

Supplementary file_1

Journal: ***Biotechnology Progress***

Manuscript title: Systemic approach for callus culture stage optimization – Using *Capsicum baccatum* L.var. pendulum as example

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Supplementary file_1. Primary data derived from multilevel factorial design. The results are expressed as mean ± standard deviation. They were ordered taking into account the explant's origin, from root to cotyledon and keeping the sequence showed in Table 1. Superscript numbers (^{1,2,3}) represent the number of repetitions of each experimental run and * represents samples without callus formation.

Run	PGR Concentracion (mg.L^{-1})	Callus Mass (mg)	Budding Index (%)	Antioxidant Activity (%)	Total Phenols (mg of GAE/g DE)	Flavonoids (mg of RU/g DE)
1	0.023	*^{3}	*^{3}	*^{3}	*^{3}	*^{3}
2	1.138	144,00±146.80^{2}	40.00±28.28^{2}	1.91^{1}	0.36^{1}	1.80^{1}
3	2.253	80.90±84.43^{2}	60.00±28.28^{2}	3.40^{1}	0.00^{1}	1.80^{1}
4	5.000	*^{3}	*^{3}	*^{3}	*^{3}	*^{3}
5	10.000	*^{2}	*^{2}	*^{2}	*^{2}	*^{2}
6	0.023	*^{3}	*^{3}	*^{3}	*^{3}	*^{3}
7	1.117	225.00±156.83^{3}	46.67±11.55^{3}	41.48±24.72^{3}	13.13±8.86^{3}	20.44±16.38^{3}
8	2.210	205.05±31,75^{2}	70.00±14.14^{2}	21.67±11.19^{2}	6.55±4.11^{2}	10.47±11.09^{2}
9	5.000	123.15±13.51^{2}	60.00±28.28^{2}	1.14±1.11^{2}	2.45±0.30^{2}	3.25±1.45^{2}
10	10.000	90.00±34.08^{2}	40.00±0.00^{2}	10.82^{1}	7.15^{1}	3.80^{1}
11	0.023	267.30±95.18^{2}	80.00±0.00^{2}	5.50±0,59^{2}	1.12±1.16^{2}	1.14±0.24^{2}
12	1.138	498.23±165.88^{3}	86.67±11.55^{3}	7.28±0.46^{3}	1.66±0.86^{3}	1.40±0.67^{3}
13	2.253	104.83±29.25^{3}	40.00±0.00^{3}	4.43±0.36^{2}	1.18±0.99^{2}	1.61±0.12^{2}

14	5.000	*[1]	*[1]	*[1]	*[1]	*[1]
15	10.000	*[2]	*[2]	*[2]	*[2]	*[2]
16	0.023	*[2]	*[2]	*[2]	*[2]	*[2]
17	1.138	152.93±27.31[3]	53.33±11.55[3]	7.30±1.24[3]	2.00±0.00[2]	2.53±0.45[3]
18	2.253	181.10±56.69[3]	66.67±11.55[3]	3.04±1.55[3]	0.00[3]	0.80±0.44[3]
19	5.000	57.93±54.78[3]	20.00±20.00[3]	3.58[1]	0.00[1]	11.97[1]
20	10.000	*[3]	*[3]	*[3]	*[3]	*[3]
21	0.023	*[3]	*[3]	*[3]	*[3]	*[3]
22	1.117	203.30±131.27[3]	53.33±11.55[3]	32.52±25.04[3]	10.00±7.37[3]	16.09±15.86[3]
23	2.210	312.65±196.36[2]	60.00±0.00[3]	9.56±7.30[2]	2.67±3.51[2]	2.35±1.27[2]
24	5.000	324.50[1]	80.00[1]	3.35[1]	2.73[1]	1.59[1]
25	10.000	124.30±3.96[2]	60.00±0.00[2]	6.09[1]	5.03[1]	1.93[1]
26	0.023	302.85±51.83[2]	80.00±0.00[2]	8.58±0.12[2]	0.27±0.04[2]	1.48±1.22[2]
27	1.138	862.43±114.48[3]	93.33±1.55[3]	7.58±4.07[3]	2.77±1.19[3]	2.58±0.58[3]
28	2.253	552.90±123.16[3]	86.67±11.55[3]	1.47±0.80[3]	0.40±0.35[3]	1.38±0.07[3]
29	5.000	*[1]	*[1]	*[1]	*[1]	*[1]
30	10.000	*[1]	*[1]	*[1]	*[1]	*[1]
31	0.023	37.13±64.32[3]	6.67±11.55[3]	3.82[1]	0.27[1]	1.17[1]
32	1.138	372.93±120.80[3]	66.67±11.55[3]	11.19±3.47[3]	3.07±0.97[3]	3.18±0.72[3]
33	2.253	519.13±203.15[3]	93.33±11.55[3]	4.90±1.02[3]	2.48±1.32[3]	1.20±0.22[3]
34	5.000	464.80±150.33[2]	70.00±14.14[2]	3.11±0.20[2]	1.21±0.09[2]	1.59±0.29[2]
35	10.000	190.73±98.25[3]	60.00±20.00[3]	4.54±0.72[2]	1.33±0.6[2]	2.00±0.00[2]
36	0.023	*[3]	*[3]	*[3]	*[3]	*[3]
37	1.117	280.20±223.04[3]	40.00±20.00[3]	20.84±0.72[2]	6.48±1.97[2]	8.89±5.24[2]
38	2.210	137.10±10.32[2]	40.00±0.00[2]	3.06±0.05[2]	0.15±0.21[2]	0.93±0.24[2]
39	5.000	252.10±1.13[2]	60.00±0.00[2]	3.49±0.14[2]	0.76±1.07[2]	1.87±0.29[2]
40	10.000	135.50[1]	60.00[1]	0,00[1]	0.00[1]	2.07[1]
41	0.023	229.60[1]	60.00[1]	4.77[1]	0.36[1]	1.45[1]
42	1.138	253.55±46.74[2]	60.00±0.00[2]	3.56±1.60[2]	1.82±1,20[2]	1.10±0.78[2]
43	2.253	460.67±482.34[3]	73.33±23.09[3]	3.53±2.26[3]	1.72±0.21[3]	2.67±1.48[3]
44	5.000	*[2]	*[2]	*[2]	*[2]	*[2]
45	10.000	125.93±71.25[3]	33.33±11.55[3]	0.41±0.58[2]	0.24±0.34[2]	4.10 ±3.11[2]

CONCLUSÃO

Foi possível a padronização dos explantes por meio de um delineamento experimental fatorial completoa dois níveis (2^4) no qual a melhor combinação de tratamentos para germinação *in vitro* e desenvolvimento de plântulasquanto ao tipo de solução para condicionamento osmótico e meio germinativo foram água + ágar e água + ágar-ácido giberélico (GA_3)para os genótipos Pitanga e Cambuci, respectivamente.

Os calos derivados de explantes do genótipo Cambuci – *Capsicum baccatum* var.pendulum apresentaram diferentes respostas para o delineamento fatorial multiníveis, no qual, os valores obtidos nas condições otimizadas foram: massa de calo (225,03 mg), atividade antioxidante (35,95%), fenóis totais (11,48 mg de GAE / g DE) e flavonóides (15,92 mg de RU / g DE), superando em termos de atividade antioxidante valores previamente relatados para esta espécie quando cultivada naturalmente.