

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL  
CENTRO DE BIOTECNOLOGIA  
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E  
MOLECULAR

**QUANTIFICAÇÃO DE HORMÔNIOS DURANTE A DORMÊNCIA DE GEMAS  
DE MACIEIRA**

Dissertação de Mestrado

**Julio de Andrade Garighan**

Porto Alegre, Dezembro de 2017

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL  
CENTRO DE BIOTECNOLOGIA  
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E  
MOLECULAR

**QUANTIFICAÇÃO DE HORMÔNIOS DURANTE A DORMÊNCIA DE GEMAS  
DE MACIEIRA**

**Julio de Andrade Garighan**

Dissertação submetida ao programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da UFRGS como requisito parcial para a obtenção do título de Mestre

Orientador: Prof. Dr. Giancarlo Pasquali  
Coorientadores: Dr. Luís Fernando Revers  
Dr. Henrique Pessoa dos Santos

Porto Alegre, Dezembro de 2017

Este trabalho foi desenvolvido nos Laboratórios de Genética Molecular Vegetal e de Fisiologia Vegetal da Embrapa Uva e Vinho, Bento Gonçalves, RS. Recebeu auxílio financeiro da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), da Financiadora de Estudos e Projetos (FINEP) e da Empresa Brasileira de Pesquisa Agropecuária (Embrapa).

## Agradecimentos

*A Deus, pela oportunidade de vida.*

*Em especial à minha família: meus pais Beatriz e Carlos, que sempre incentivaram meus estudos, fornecendo as condições necessárias para eu alcançar meus objetivos; e minhas irmãs Gabriella, Bibiana e ao Ygor, que apesar da distância sempre me motivaram a seguir em frente e não desistir dos meus sonhos*

*Ao Dr. Henrique Pessoa dos Santos, responsável pelo Laboratório de Fisiologia Vegetal da Embrapa Uva e Vinho, por sua orientação, ensino e entusiasmo ao longo deste mestrado, principalmente pela confiança em mim depositada e pelos conhecimentos compartilhados. As produtivas discussões sempre agregaram conhecimento e ajudaram a manter o foco durante a execução desse trabalho.*

*Ao Dr. Luís Fernando Revers, responsável pelo Laboratório de Genética Molecular Vegetal da Embrapa Uva e Vinho, por sua coorientação, apoio e confiança em mim depositada, além dos conhecimentos compartilhados.*

*Ao meu terceiro orientador, Prof. Dr. Giancarlo Pasquali, por sua coorientação, confiança em meu potencial e entusiasmo com o projeto.*

*À Universidade Federal do Rio Grande do Sul, pela oportunidade de estudo em uma universidade pública e de qualidade.*

*À Embrapa Uva e Vinho, por permitir acesso à sua estrutura e fornecer os recursos necessários para a realização desse trabalho.*

*À CAPES, pela concessão da bolsa de Mestrado.*

*Aos colegas do Laboratório de Fisiologia Vegetal, Daniel, Aline, Tiago, Vagner e Suelen, pelo apoio, conversas, amizade e carinho.*

*Aos colegas do Laboratório de Genética Molecular Vegetal, Caru, Rafael, Vanessa, Jaiana, Yohanna, Regina, Amanda, Tiago, Lariane, Danieli e Roberta, pela amizade e carinho.*

*Aos meus queridos amigos Bianca, Gabriela, Jackson, Tais, Mariana, Thais, Raissa e Patrícia por acompanharem toda essa aventura desde a graduação, vocês foram essenciais.*

*Ao PPGBCM e ao CBiot, pela qualidade de ensino, especialmente à minha Comissão de Acompanhamento, Márcia Márgis-Pinheiro e Arthur Fett, pela atenção despendida durante estes anos, e à Sílvia e ao Luciano, pela agilidade e prontidão.*

*Aos Professores da Banca Examinadora, pela disponibilidade, pelas críticas e sugestões.*

## Sumário

|  |            |
|--|------------|
| <b>LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES .....</b>  | <b>6</b>   |
| <b>RESUMO.....</b>   | <b>8</b>   |
| <b>ABSTRACT.....</b>   | <b>9</b>   |
| <b>1 INTRODUÇÃO .....</b>  | <b>10</b>  |
| 1.1 DORMÊNCIA.....   | 10         |
| 1.2 REGULAÇÃO HORMONAL NA DORMÊNCIA .....  | 12         |
| 1.3 MÉTODO DE ANÁLISE DE HORMÔNIOS.....  | 13         |
| 1.3.1 EXTRAÇÃO HORMONAL .....  | 15         |
| 1.3.2 QUANTIFICAÇÃO HORMONAL.....  | 16         |
| 1.4 EFEITO MATRIZ E ESTRATÉGIAS DE CONTROLE.....   | 17         |
| 1.4.1 PURIFICAÇÃO HORMONAL.....  | 18         |
| 1.5 CULTURA DA MACIEIRA .....  | 22         |
| <b>2 OBJETIVOS.....</b>  | <b>25</b>  |
| 2.1 OBJETIVO GERAL .....   | 25         |
| 2.2 OBJETIVOS ESPECÍFICOS.....   | 25         |
| <b>3 CAPÍTULO I.....</b>   | <b>26</b>  |
| A protocol for hormone extraction and quantification from complex lignified<br>plant bud tissues. ....     | 26         |
| <b>4 CAPÍTULO II.....</b>  | <b>70</b>  |
| Hormonal Quantification in Dormant Buds of Apple Trees under the Influence<br>of Different Rootstocks..... | 70         |
| <b>5 DISCUSSÃO .....</b>   | <b>97</b>  |
| 5.1 MÉTODO PARA EXTRAÇÃO, PURIFICAÇÃO E<br>DETECÇÃO/QUANTIFICAÇÃO DE HORMÔNIOS EM GEMAS<br>DORMENTES. .... | 97         |
| 5.2 BALANÇO HORMONAL DE GEMAS DORMENTES DE MACIEIRA SOB<br>INFLUÊNCIA DE DIFERENTES PORTA-ENXERTOS.....    | 101        |
| <b>6 PERSPECTIVAS.....</b>   | <b>107</b> |
| <b>7 REFERÊNCIAS BIBLIOGRÁFICAS .....</b>  | <b>108</b> |
| <b>CURRICULUM VITÆ resumido.....</b>   | <b>113</b> |
| <b>APÊNDICE.....</b>   | <b>117</b> |

## LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

- ABA - ácido abscísico, do inglês, *abscisic acid*
- APCI – ionização química à pressão atmosférica, do inglês *Atmospheric Pressure Chemical Ionization*
- ARR-tipo B - domínio de ligação ao DNA, associado a respostas a citocinina, do inglês *Arabidopsis thaliana Response Regulator*
- BB – brotação da gema apical acima de 50%, do inglês *Bud Break*
- Castel – cultivar Castel Gala de macieira
- CH – hora(s) de resfriamento, do inglês, *Chilling Hour(s)*, período(s) sob temperaturas abaixo ou igual a 7,2°C
- CKs – citocininas, do inglês *cytokinin*
- CL – cromatografia líquida
- CR – requerimento de frio, do inglês, *Cold Requirement*
- cv. - cultivar
- DAM – gene MADS-BOX associados a dormência, do inglês *DORMANCY-ASSOCIATED MADS-BOX (DAM) genes*
- DNA - ácido desoxirribonucleico, do inglês *Deoxyribonucleic Acid*
- ESI – ionização por eletropulverização, do inglês, *Eletrospray Ionization*
- FAO – Organização das Nações Unidas para Alimentação e Agricultura, do inglês *Food and Agriculture Organization*
- g - gravidade
- GA - ácido giberélico, do inglês, *gibberellic acid*
- Gala – cultivar Gala Standard de macieira
- GA<sub>3</sub> - ácido giberélico 3, do inglês, *gibberellic acid 3*
- GA<sub>4</sub> - ácido giberélico 4, do inglês, *gibberellic acid 4*
- GH17 - 1,3-β-glicanases 17
- ha - hectare
- IAA – ácido indol-3-acético, do inglês, *indolacetic acid*
- JA – ácido jasmônico, do inglês, *jasmonic acid*
- LLE – extração líquido-líquido ou em fase líquida, do inglês, *Liquid Phase Extraction*
- MADS box – motivo de ligação ao DNA, do acrônimo em inglês de *MCM1 AGAMOUS DEFICIENS SRF*
- MaM9 – Porta-enxerto Marubakaido com interenxerto de M9 de 10 cm
- Maruba – Porta-enxerto Marubakaido (*Malus prunifolia*)
- M9 – Porta-enxerto East Malling 9 (*Malus pumilla*)
- ME – efeito matriz, do inglês, *Matrix Effect*
- MRM - monitoramento de reações múltiplas, do inglês *multiple-reaction monitoring*
- MS – espectrometria de massas, do inglês, *Mass Spectrometry*
- m/z – medida de ionização da massa por carga

Neg – modo de ionização negativa no cone, localizado entre a ionização e os espectrômetros de massa

pH – potencial de hidrogênio, do inglês *Potential of Hydrogen*

pKa - constante de dissociação ácida

Pos – modo de ionização positiva no cone, localizado entre a ionização e os espectrômetros de massa

PVPP – polivinilpolipirrolidona

QuEChERS - técnica de análise desenvolvida originalmente para resíduos de pesticidas, nomeada de acordo com o acrônimo em inglês de *Quick, Easy, Cheap, Effective, Rugged, and Safe*

RNA - ácido ribonucleico, do inglês *Ribonucleic Acid*

RT-qPCR – reação em cadeia da polimerase quantitativa em tempo real, do inglês *Real-Time Quantitative Polymerase Chain Reaction*

SA – ácido salicílico, do inglês, *salicylic acid*

SLV – guia de regras de validação de métodos, do inglês *Guidelines for Single Laboratory Validation*

S/N – razão sinal/ruído, do inglês *Signal/Noise*

SPE – extração em fase sólida, do inglês, *Solid Phase Extraction*

ton - tonelada

UPLC – cromatografia líquida de ultradesempenho, do inglês, *Ultra-Performance Liquid Chromatography*

UPLC-MS/MS – aparelho de UPLC acoplado diretamente a um aparelho de espectrometria de massas em tandem

v/v – volume por volume

V - volume

Z – zeatina

## RESUMO

A macieira (*Malus x domestica* Borkh.) é uma frutífera de grande importância econômica que apresenta dormência invernal. Sua produtividade depende da superação da dormência, a qual é dependente de um somatório de períodos de frio hibernal. Este somatório é variável entre genótipos. Análises gênicas permitiram demonstrar que as vias de síntese de hormônios são diferencialmente ativadas ou reprimidas na dormência e na brotação de macieiras e outros vegetais. Apesar do avanço na compreensão da regulação gênica deste mecanismo, ainda existem carências da compreensão das vias de regulação de metabólitos nesta fase. A gema dormente é um material difícil de ser trabalhado por apresentar tecidos lignificados ricos em pigmentos e compostos fenólicos e com alta tendência à oxidação. Pelo presente trabalho, teve-se por foco o desenvolvimento de método de extração, purificação e detecção/quantificação de hormônios vegetais nas gemas dormentes de macieira e a utilização desse método na caracterização do balanço hormonal destas gemas. O método desenvolvido é sensível e preciso, com capacidade de análise de sete hormônios vegetais (ABA, GA<sub>3</sub>, GA<sub>4</sub>, IAA, JA, SA e Zea), tanto em gemas dormentes como em tecidos pigmentados, lignificados e com alto efeito matriz. Foram coletadas amostras de gemas de macieira ao longo da dormência de cultivares com requerimento de frio contrastante (Gala Standard e Castel Gala, requerentes de 600 e 300 horas de frio, respectivamente), sobre porta-enxertos com contrastes de vigor (alto vigor, Maruba; médio vigor, Maruba/M9; e baixo vigor, M9), ao longo de um inverno com alta oferta de frio para a região (901 CH in 2016). A modulação hormonal de gemas dormentes foi semelhante entre as cultivares mesmo com fenótipos de brotação diferentes. Não houve quantificação dos hormônios propostos no começo da endodormência. ABA e GA<sub>4</sub> foram os principais reguladores da dormência em um mecanismo muito similar ao da dormência de sementes. Porta-enxertos podem atrasar a brotação com estímulo de carga de ABA, como ocorre com M9. Isso demonstra o papel fundamental dos hormônios na dormência e como o estudo de porta-enxertos pode ajudar no desenvolvimento da pomicultura.



## ABSTRACT

The apple tree (*Malus x domestica* Borkh.) is an economically important fruit tree that presents winter dormancy. Its productivity depends on dormancy overcoming, which is dependent on a sum of periods of chilling during winter. This sum is variable among genotypes. Genetic analyses allowed to show that hormonal biosynthetic pathways are differentially activated or repressed in dormancy and sprouting in apple trees and other plants. Despite the advances in the understanding of the genetic regulation during this mechanism, there is still a lack of knowledge about relevant metabolic pathways in this phase. The dormant bud is a difficult material to work with since it presents lignified tissues with pigments and phenolic compounds that are highly susceptible to oxidation. The present work focused on the development of a method for the extraction, purification and detection/quantification of plant hormones in apple dormant buds and the use of this method to characterize the hormonal balance in these buds. The developed method is sensitive and accurate, allowing the analyses of seven plant hormones (ABA, GA<sub>3</sub>, GA<sub>4</sub>, IAA, JA, SA and Zea) in dormant buds or in pigmented, lignified tissues highly susceptible to matrix effect. Samples of apple buds were collected along the dormancy period from cultivars with contrasting cold requirements (Gala Standard and Castel Gala, which require 600 and 300 chilling hours, respectively), on rootstocks with contrasting vigor (high vigor, Maruba; medium vigor, Maruba/M9; and low vigor, M9), during a particularly cold winter to the region (901 CH in 2016). The hormonal modulation of dormant buds was similar among cultivars even with different bud break phenotypes. There was no quantification of the proposed hormones at the beginning of endodormancy. ABA and GA<sub>4</sub> were the main regulators of dormancy in a cold year by a mechanism very similar to that found in seed dormancy. Rootstocks may delay bud break with ABA loading stimulus, as occurs with M9. This demonstrates the key role of hormones in dormancy and how the study of rootstocks may help the development of pomiculture.

# 1 INTRODUÇÃO

## 1.1 DORMÊNCIA

As plantas perenes enfrentam variações climáticas sazonais em diversas amplitudes e intensidades. Dentro deste grupo, as frutíferas de clima temperado são as mais importantes tanto do ponto de vista econômico como pelas adaptações desenvolvidas a estas variações (FALAVIGNA *et al.*, 2015). A dormência surge como uma adaptação destas plantas ao frio invernal de alta intensidade, sendo a quantidade e qualidade da produção frutífera dependente do ciclo dormente anterior (SINGH *et al.*, 2017). Como uma adaptação ao clima, principalmente ao frio, há a formação de gemas dormentes (ROHDE & BHALERAO, 2007). A resposta fenológica da planta a essas mudanças é conhecida, porém há pouco entendimento sobre os processos bioquímicos associados a este mecanismo e como a variação ambiental é percebida e traduzida no estado de dormência (ROHDE & BHALERAO, 2007; RINNE *et al.*, 2011; SINGH *et al.*, 2017). Estudos gênicos baseados em transcritômica (*RNA-Seq*) têm trazido entendimento sobre ativação de rotas metabólicas e o papel de grupos de genes na indução, na manutenção e na liberação da dormência. Tem sido demonstrado o papel de genes na regulação do metabolismo como os repressores *DAM* ("*Dormancy-associated MADSbox*") e, principalmente, suas interações com vias de síntese de hormônios vegetais (WISNIEWSKI *et al.*, 2015; TUAN *et al.*, 2017). Porém, esses avanços têm progredido muito no nível gênico e pouco no nível metabólico, com as indicações de relação de metabólitos sendo confirmados somente em alguns processos (TWORKOSKI & FAZIO, 2016; TUAN *et al.*, 2017). A maioria desses avanços no conhecimento de metabólitos é para aqueles relacionados com a proteção da gema ao frio e à dessecação (LEIDA *et al.*, 2010; BAI *et al.*, 2013; FALAVIGNA *et al.*, 2014; FALAVIGNA *et al.*, 2015; PORTO *et al.*, 2015). Devido a estes resultados e à importância em diversos processos fisiológicos, surge então a necessidade de uma caracterização do balanço hormonal na dormência das gemas. Este enfoque hormonal serve tanto para verificar se a ativação das vias de síntese e degradação são compatíveis com os eventos fenológicos, como também a sua

relação dentro do processo da dormência. Entretanto, por ser um conjunto de tecidos pouco trabalhado nesse tipo de análise quantitativa, as gemas necessitam do desenvolvimento de um método próprio para a correta extração e detecção/quantificação desses analitos. O desenvolvimento do método é parte do trabalho desta dissertação e é mostrado no Capítulo I.

As frutíferas de clima temperado apresentam o estado de dormência para superar as condições ambientais adversas de inverno, principalmente de temperaturas congelantes que limitam o desenvolvimento normal e a sobrevivência. Na entrada da dormência, ocorre a queda das folhas, a parada de crescimento do meristema apical e o encapsulamento desse meristema por escamas formadas por folhas diferenciadas (ARORA *et al.*, 2003; RINNE *et al.*, 2011). Neste processo de isolamento do meristema, ocorre o acúmulo de placas de calose nos plasmodesmatas para proteção frente ao frio e à seca (ARORA *et al.*, 2003; RINNE *et al.*, 2011). No caso específico da família *Rosaceae*, essa entrada da dormência ocorre em resposta ao frio (ROHDE & BHALERAO, 2007). A dormência hiberna de gemas é classificada em endodormência e ecodormência. A primeira é a incapacidade de brotar mesmo em condições ambientais propícias, coordenadas por fatores endógenos da gema e, a segunda, é a inibição da brotação por condições ambientais ou fatores externos à planta (ROHDE & BHALERAO, 2007). Há também a paradormência, que é a inibição da brotação por outros tecidos, principalmente por dominância apical (ROHDE & BHALERAO, 2007). Neste trabalho visa-se estudar a dormência hiberna de gemas apicais. Assim, somente serão considerados e discutidos a endodormência e a ecodormência.

A indução, a manutenção e a superação da endodormência está relacionada ao tempo de exposição ao frio, referenciado como temperaturas iguais ou abaixo de 7,2°C. Quando o somatório de frio é atingido, as gemas superam o estado de endodormência, passando para o estado de ecodormência se o ambiente não for favorável como, por exemplo, em primaveras frias ou sob deficiência hídrica (ROHDE & BHALERAO, 2007; SINGH *et al.*, 2017). Esse requerimento ou soma de frio para superar a endodormência é uma característica precisa, diretamente associada ao genoma e com grandes contrastes entre espécies e cultivares (LANG *et al.*, 1987; FALAVIGNA *et al.*, 2015). Há diversos indícios da atuação hormonal

tanto na entrada quanto na manutenção e na liberação da endodormência, porém eles ainda são pouco elucidados (PORTO *et al.*, 2016; KUMAR *et al.*, 2017; TUAN *et al.*, 2017).

## 1.2 REGULAÇÃO HORMONAL NA DORMÊNCIA

O ácido abscísico (ABA, do inglês, *abscisic acid*) e o ácido giberélico (GA, do inglês, *gibberellic acid*) são definidos como os reguladores da dormência de sementes e dados de expressão gênica de sementes e gemas dormentes de pereira mostraram a ativação de vias metabólicas correlatas, indicando que esses hormônios podem agir de forma semelhante na dormência de gemas (WANG *et al.*, 2016). O ABA está ligado à repressão do metabolismo, à resposta a estresses bióticos/abióticos e à adaptação. Já foi descrito como indutor da formação da gema (RUTTINK *et al.*, 2007). O ABA foi quantificado nas gemas dormentes de pera e foi demonstrado um aumento do hormônio na transição dos estados de endo- para ecodormente, com queda da concentração logo em seguida (TUAN *et al.*, 2017). Foi demonstrado também a relação de ABA com processos de desidratação ou resposta ao frio e, mais recentemente, a relação entre ABA e os genes *DAM* na liberação da endodormência (WILKINSON & DAVIES, 2010; WANG *et al.*, 2016; TUAN *et al.*, 2017).

Depois de ABA, o GA é outro hormônio que se espera ter papel importante na dormência, já que está relacionado aos processos de expansão celular e crescimento. Há indícios de que a presença de GA diminui na entrada da dormência assim como sua via de síntese tem atividade diminuída (RINNE *et al.*, 2011). Outro indício de que GA está envolvido na dormência é o fato de enzimas 1,3- $\beta$ -glicanases 17 (GH17) serem recrutadas por GA durante o desmantelamento da barreira de calose, tornando a gema acessível ao resto da planta e favorecendo a brotação (RINNE *et al.*, 2011). Além disso, dados de expressão gênica mostraram a ativação da via de síntese de GA durante a etapa de brotação das gemas em macieira (KUMAR *et al.*, 2017).

Uma terceira classe hormonal que tem recebido atenção no contexto da dormência é o das citocininas (CKs, do inglês, *cytokinins*), em particular a

zeatina (Zea). ABA e GA são apontados como fundamentais no processo da dormência quando as condições de acúmulo de frio hibernar são satisfatórias. Porém, quando não há o suprimento de frio suficiente, as rotas metabólicas de resposta a Z são ativadas. Neste sentido, foram encontrados elementos *cis* para ARR-tipo B nos promotores dos genes *DAM*, mostrando uma relação da Z na reativação do metabolismo reprimido e sua liberação em condições de falta de frio (HOOIJDONK *et al.*, 2011; KUMAR *et al.*, 2017).

A interligação na regulação do crescimento por auxinas e GA já é conhecida (ROSS *et al.*, 2011), assim como as participações de CKs, auxinas e ABA na dominância apical (DUN *et al.*, 2009). Além disso, as CKs e as auxinas autorregulam-se e foram apontadas como tendo suas vias de síntese ativadas durante a dormência e a brotação da maçã (FALAVIGNA *et al.*, 2015; KUMAR *et al.*, 2017).

Todos esses avanços no conhecimento de expressão gênica associados ao metabolismo hormonal levantam a necessidade de um monitoramento direto de todos esses hormônios para as análises, não só dos seus papéis sobre o metabolismo da gema, mas também de suas inter-relações no processo da dormência. Os dados de quantificação desses hormônios podem explicar alguns fenômenos da dormência de gemas, já que processos complexos de vegetais superiores apresentam diversos níveis de regulações e a participação de diversas rotas metabólicas.

### 1.3 MÉTODO DE ANÁLISE DE HORMÔNIOS

Com o desenvolvimento de métodos e de serviços terceirizados de alto desempenho (*high-throughput*) para análises gênicas, particularmente a transcritômica, houve um grande avanço na produção de informações, no entendimento da regulação gênica e na formulação de hipóteses sobre o funcionamento de diversos processos fisiológicos. Essas hipóteses baseiam-se na análise de transcrição diferencial, indicando quais rotas ou processos estão 'ativos' no estado analisado (KUMAR *et al.*, 2017; TUAN *et al.*, 2017). Os avanços obtidos com tais processos foram úteis no delineamento do foco de estudo e na compreensão geral dos processos envolvidos. Contudo, isso ainda

pode sofrer modulação nas diversas etapas regulatórias que existem durante a transcrição e a tradução (GEBAUER & HENTZE, 2004; HOY, 2013). Há mecanismos de intensificação, inativação de um metabólito ou de marcação para estocagem para ser reativado muito posteriormente sem nenhum sinal de ativação da via metabólica (BAJGUZ & PIOTROWSKA, 2009). Com todas essas possibilidades de alterações entre o que está transcricionalmente ativo e o que acontece fenologicamente, a detecção da molécula ativa metabolicamente torna-se fundamental para confirmar as ativações de vias transcricionais e esclarecer o efeito fenológico observado.

A análise hormonal tem sido utilizada para a caracterização de fenômenos fisiológicos e tem contribuição fundamental para o avanço no conhecimento destes processos regulatórios. Os métodos de quantificação hormonal não têm passado pelos mesmos processos de *high-throughput* que os métodos gênicos, a exemplo da reação em cadeia quantitativa da DNA polimerase precedida de transcrição reversa (RT-qPCR). Outros limitantes, como a incapacidade de se amplificar o número de cópias do analito metabólico (como por PCR para DNA) ou a inexistência de meios de extração específicos de hormônios (como o que ocorre com DNA e RNA) dificultam a análise hormonal. O hormônio extraído da amostra, que geralmente está em baixa quantidade, deve competir com outras moléculas para ser quantificado, o que torna a quantificação hormonal um processo por si só mais difícil que os correlatos gênicos. A partir desse tipo de necessidade, surgiram meios de quantificação de alta sensibilidade, como a cromatografia líquida de ultra performance acoplado à espectrometria de massas em tandem (UPLC-MS/MS, do inglês, *Ultra Performance Liquid Chromatography - tandem mass spectrometer*), que são capazes de separar as moléculas por sua afinidade iônica na cromatografia líquida de alta pressão e essas moléculas de mesmo comportamento iônico são segregadas de acordo com a massa original (massa mãe) no primeiro quadrupolo do espectrômetro de massas, sofrem uma clivagem em um quadrupolo intermediário e as moléculas-fragmentos (massas filhas) são selecionadas no terceiro. Esse processo garante a seleção por comportamento iônico (na UPLC) e massa (na MS/MS), sendo altamente

seletivo e sensível para detecção e quantificação de metabólitos em amostras complexas (CHAMBERS *et al.*, 2007; NIU *et al.*, 2014; CUI *et al.*, 2015).

### 1.3.1 EXTRAÇÃO HORMONAL

A extração dos hormônios é um processo baseado na mistura da amostra com uma solução de extração por um determinado período, seguido de posterior separação da amostra e da solução de extração com o(s) analito(s) alvo(s), geralmente já sendo encaminhado(s) para a análise qualitativa e quantitativa (DOBREV & KAMÍNEK, 2002; ZHOU *et al.*, 2003). Experimentações no modo de preparo da amostra e misturas com a solução de extração, tais como tempos de incubação, pulverizações para aumento da área de contato e rompimento das barreiras mais difíceis como parede celular vegetal, já foram desenvolvidas (DOBREV & KAMÍNEK, 2002; MÜLLER & MUNNÉ-BOSCH, 2011; CUI *et al.*, 2015).

A solução de extração é outro ponto que tem sido trabalhado, sendo álcoois (metanol e isopropanol), acetonitrila e água (em menor proporção) os principais solventes empregados em análises de hormônios vegetais (DOBREV & VANKOVA, 2012; HAN *et al.*, 2012; GILAR *et al.*, 2014; CUI *et al.*, 2015). Junto com os solventes, há também o emprego de ácidos orgânicos para a estabilização das amostras, geralmente ácido fórmico e acético (DOBREV & VANKOVA, 2012; HAN *et al.*, 2012; GILAR *et al.*, 2014; CUI *et al.*, 2015). Há descrições de que certos hormônios seriam extraídos preferencialmente por certas combinações de solventes extratores, como ABA, GA e JÁ sendo preferencialmente extraídos por isopropanol enquanto IAA e AS o seriam por acetonitrila, porém isso não é constante nas diversas amostras vegetais (CUI *et al.*, 2015).

Gemas dormentes de macieira apresentam grande quantidade de compostos fenólicos, lignina e outros interferentes no contexto de uma análise hormonal (SZECSKÓ *et al.*, 2004; BJO *et al.*, 2007; LJUNG *et al.*, 2010). Tais compostos impedem a utilização direta, em gemas dormentes, dos métodos de extração hormonal de passo único, comumente utilizados em tecidos moles e

menos complexos, como folhas de *Arabidopsis thaliana*. Tais métodos de extração extraem diversos outros metabólitos além de hormônios, requerendo um método de detecção altamente seletivo para quantificá-los. Estes outros metabólitos atrapalham a detecção do analito hormonal, o que torna impreciso a quantificação do mesmo (MATUSZEWSKI & CONSTANZER, 1998; MATUSZEWSKI *et al.*, 2003; CHAMBERS *et al.*, 2007; TRUFELLI *et al.*, 2011).

### 1.3.2 QUANTIFICAÇÃO HORMONAL

A combinação da ionização por eletropulverização (ESI, do inglês, *Electrospray Ionization*) com UPLC-MS/MS (UPLC-ESI-MS/MS) é uma técnica de quantificação que se baseia na separação dos analitos por afinidade iônica na cromatografia líquida e a seleção/identificação dos analitos pelas suas massas-mães, padrões de fragmentações e fragmentos dominantes (massas-filhas) antes da fase de detecção. Com essas características, essa técnica comporta uma alta capacidade de seleção, sensibilidade e especificidade analítica, tornando-se uma técnica padrão para análise de compostos de baixa concentração em amostras complexas (MATUSZEWSKI & CONSTANZER, 1998; LAMBERT & MURPHY, 2003). Apesar destas vantagens que agregam precisão e exatidão, esta técnica apresenta alguns problemas na sua etapa de ionização, durante a transição da fase líquida para fase gasosa no espectrômetro de massa. Na ESI, ocorre a pulverização do líquido proveniente da UPLC em uma atmosfera ionizada sob alta temperatura. Nesse ambiente, ocorre a vaporização dos eluentes e a ionização dos compostos (solutos), pois somente no estado ionizado é que as cargas podem ser atraídas para o cone de acesso para os quadrupolos de separação de massas (MS) e o detector. Portanto, qualquer composto que possua as mesmas propriedades físico-químicas de interação com a coluna da UPLC e que, por consequência, coelue com os analitos-alvos, pode interferir nesse processo de ionização, impedindo que o analito-alvo seja capturado para a detecção e quantificação, gerando um resultado falso-negativo. Este processo de interferência de outros metabólitos da amostra sobre os analitos-alvos é denominado de efeito matriz (ME, do inglês, *Matrix Effect*; CHAMBERS *et al.*, 2007; TRUFELLI *et al.*, 2011).



## 1.4 EFEITO MATRIZ E ESTRATÉGIAS DE CONTROLE

O ME é um sério interferente nas análises que utilizam cromatógrafos líquidos acoplados a espectrômetros de massas, e tem se tornado mais importante com o avanço da utilização de métodos quantitativos em amostras mais complexas que não eram utilizadas anteriormente. A partir da evidência desse problema e a percepção de sua presença em muitas amostras, foi desenvolvido uma padronização ou um limite de aceitação para a presença de ME. A *Guidelines for Single Laboratory Validation (SLV)* define que ME só é importante na análise quando atinge um limite superior a 10% de interferência na recuperação de padrão adicionado (AOAC, 2002; MATUSZEWSKI *et al.*, 2003; TRUFELLI *et al.*, 2011). Para analisar tal interferência, é necessário que o equipamento esteja calibrado com a curva padrão corretamente montada, sendo, neste caso, a utilização da matriz (amostra) como parte da curva padrão (BOSCO *et al.*, 2014). O ME afeta a reprodução e a precisão da análise, reduzindo a intensidade iônica dos analitos (MATUSZEWSKI *et al.*, 2003). O ME tem impacto maior em ionização por ESI que por ionização química sob pressão atmosférica (APCI, do inglês, *Atmospheric Pressure Chemical Ionization*), especialmente quando o ESI estiver configurado no modo de ionização positiva (ESI+). Entretanto, as duas técnicas são suscetíveis às interferências da matriz (LAMBERT & MURPHY, 2003).

O ME foi reportado inicialmente em amostras clínicas, na análise de reprodutibilidade de meios de quantificação de finasterida em plasma humano (MATUSZEWSKI & CONSTANZER, 1998). Desde então, um grande número de tentativas de eliminação do ME foram desenvolvidos (MATUSZEWSKI *et al.*, 2003; CHAMBERS *et al.*, 2007). Entretanto, somente a mitigação dos efeitos foi alcançado, com o ajuste de métodos de correção. Uma das soluções mais fáceis e rápidas que foi ajustada é o emprego da curva padrão em mistura com a matriz, utilizando o extrato da amostra como solvente dos padrões hormonais. Outro meio de mitigação do ME é a modulação do gradiente de solvente na cromatografia líquida, uma vez que o ME depende majoritariamente do tempo de retenção do analito-alvo e dos interferentes (AKE *et al.*, 2001). Com o ajuste de solvente e, conseqüentemente, da polaridade da

eluição, é possível obter-se variações na separação dos componentes interferentes e favorecimentos na detecção e na quantificação, porém seus efeitos são limitados a pequenas interferências do ME e não impedem falsos-negativos.

Uma estratégia comum para a mensuração e a correção do ME é com a utilização de padrões internos dos analitos-alvos. Quantidades conhecidas do padrão interno são aplicados junto com a amostra na solução de extração logo no começo do processo e sua recuperação é calculada nas etapas de detecção/quantificação. A diferença entre a quantidade aplicada e a quantidade recuperada é calculada e usada para corrigir a quantificação do analito endógeno, porém esse método não evita falsos negativos, limitando seu uso a mensuração do ME e às análises da eficiência de recuperação e eficiência do processo (VATS *et al.*, 2016). A análise de recuperação da aplicação de padrão interno no começo da extração retorna a eficiência do processo, enquanto a análise da recuperação quando o padrão interno é aplicado na amostra logo antes da leitura retorna o ME. A comparação é realizada considerando as áreas do padrão do analito em uma solução 'branca' (solução de leitura) e a área da amostra do analito adicionado nos passos anteriores. O cálculo é realizado segundo a fórmula abaixo (MATUSZEWSKI *et al.*, 2003; TRUFELLI *et al.*, 2011):

$$ME = 100 \times \left[ \left( \frac{\text{área do padrão na solução com matriz}}{\text{área do padrão na solução de leitura}} \right) - 1 \right]$$

O valor de ME é definido como intensificador (% ME positiva ou recuperação >100%), supressora (% ME negativa ou recuperação <100%) ou não alterada (% ME = zero ou recuperação = 100%). A purificação da amostra entre a extração e a quantificação é o melhor meio de mitigar ou eliminar o ME (BYLDA *et al.*, 2014).

#### 1.4.1 PURIFICAÇÃO HORMONAL

Amostras que apresentam problemas de efeito matriz, com extratos altamente pigmentados, com carboidratos ou com outros compostos

interferentes, restringem a capacidade analítica. Este problema é mais restritivo quando os analitos alvo estão em quantidades muito baixas nas amostras, como hormônios, pois o poder de extração das soluções empregadas faz a retirada não só do analito alvo, mas também de grandes quantidades de interferentes. Visando contornar este problema e evitar falsos negativos, até o momento foram desenvolvidos diversos métodos de purificação, como filtrações, precipitação de proteínas, extração líquido-líquido (LLE, do inglês, *liquid-liquid extraction*), extração em fase sólida (SPE, do inglês, *solid phase extraction*) e QuEChERS (do inglês, *Quick, Easy, Cheap, Effective, Rugged, Safe*), (AKE *et al.*, 2001; CUI *et al.*, 2015; VATS *et al.*, 2016).

LLE baseia-se na separação de componentes considerando as diferenças de solubilidade em dois líquidos imiscíveis, normalmente água e um solvente orgânico. Este método tem a desvantagem de adicionar mais uma etapa de purificação para retirar o analito-alvo da fase separada anteriormente, além de não ser eficaz em soluções altamente contaminadas por interferentes. De modo geral, a literatura destaca que a técnica LLE tem uma recuperação inferior do padrão em relação a técnica SPE, especialmente para os hormônios ácido indolacético (IAA, do inglês, *indolacetic acid*) e GA<sub>3</sub> (CUI *et al.*, 2015). A precipitação de proteínas e a filtração são métodos simples e ineficientes nas análises de hormônios, uma vez que proteínas e partículas em suspensão não representam a principal interferência.

QuEChERS é muito utilizado na área alimentícia e tem bons resultados na eliminação de açúcares e de lipídios (HOU *et al.*, 2015), porém utiliza uma grande quantidade de amostra, superiores a 50 g, o que inviabiliza o emprego em experimentos com grande número de amostras.

Por fim, destaca-se a técnica SPE, que se baseia na separação dos compostos por fixação a algum substrato com tendência a uma interação com o analito, então o sorbente com amostra é lavada por alguma substância que não separa o analito do sorbente. Após a lavagem, há eluição do analito da coluna SPE. Este método é prático, sensível, relativamente barato e rápido, não exigindo grande quantidade de amostra (AGILENT Corp., 2009). Por esses motivos, SPE foi escolhido como a estratégia de purificação dos hormônios no presente trabalho.

As colunas SPE receberam avanços como a alta compactação, que diminui seus poros, permitindo uma filtragem de resíduos grandes (maiores que 60 µm). Outro desenvolvimento foi a ligação à sílica de compostos com grupos funcionais, que permitiram a diversificação do uso das SPE e refinaram sua seleção. As SPE podem ser definidas em SPE de Fase Normal, SPE de Fase Reversa ou SPE de Troca Iônica (SUPELCO, 1998).

SPE de Fase Normal apresenta coluna com fase estacionária polar, sendo o analito de interesse polar em um solvente apolar (acetona, solventes clorados e hexano). A fase estacionária geralmente é composta de sílica ligada a grupos polares (como Si-CN, Si-NH<sub>2</sub> e Si-Diol). A retenção do analito se dá principalmente por interações entre os grupos funcionais polares do analito e da superfície do sorbente (fase estacionária). Essas interações se dão por pontes de hidrogênio, interações dipolo-dipolo, interações π-π (*pi-pi interactions*), entre outros. A eluição do analito se dá pela passagem na coluna de um solvente que seja mais polar que o sorbente, perturbando as interações entre o analito e a fase estacionária (SUPELCO, 1998).

SPE de Fase Reversa é formada por coluna com fase estacionária apolar, sendo o analito de interesse apolar ou pouco polar em um solvente polar (soluções aquosas). A fase estacionária geralmente é composta de sílica ligada ao grupo alquil ou aril. A seleção se dá por forças de van der Waals entre o analito e a fase estacionária, quando o analito se encontra em solução polar. A eluição se dá por solução apolar capaz de perturbar as forças de van der Waals existentes entre analito e fase estacionária. LC-18 e LC-8 são sílicas monoméricas, sendo as mais utilizadas (SUPELCO, 1998).

SPE de Troca Iônica (catiônica e aniônica) apresentam coluna com afinidades iônicas para analitos carregados, geralmente em soluções aquosas. O sorbente pode ser carregado positivamente (sorbente Si-NH<sub>2</sub> ou amina quaternária alifática), para seleção de compostos aniônicos, ou carregado negativamente (sorbente ligado a grupos ácidos sulfônicos alifáticos) para compostos catiônicos. Nas colunas de troca iônica, a escolha entre os sorbentes baseia-se no balanço de pH das soluções e pKa do analito. Para as colunas de troca aniônica, há a opção do sorbente com modificação de -NH<sub>2</sub> (sílica ligada a um grupo aminopropil alifático) ou amina quaternária. O primeiro

torna a coluna uma trocadora aniônica fraca (pKa 9,8), permitindo a seleção do analito por mudanças de dois níveis de pH abaixo do pKa do analito na solução de eluição (ex.: pH 7 de um pKa 9). O sorbente -NH<sub>2</sub> é usado para isolar e recuperar ânions fortes e fracos, já que seu grupo amina pode ser neutralizado com duas unidades de pH acima do pKa. No caso da amina quaternária, o sorbente se torna extremamente forte (pKa acima de 14) e é usado para a seleção de ânions fracos (pKa acima de 2) quando se deseja recuperação, ou ânions fortes (pKa abaixo de 1) quando se deseja a eliminação deste composto. É utilizado a troca de 2 níveis de pH acima do pKa do analito nesta coluna (ex.: pH 8 de um pKa 6) (SUPELCO, 1998).

Nas colunas de troca catiônica, há dois tipos de grupos funcionais que são ligados à sílica: ácido sulfônico alifático ou ácido carboxílico alifático. Nas colunas com grupos de ácidos sulfônicos alifáticos, as colunas são fortemente ácidas (pKa abaixo de 1) e são usadas para selecionar compostos fortemente catiônicos (pKa acima de 14), quando se deseja a eliminação, ou catiônicos fracos (pKa menor de 12), quando se deseja a recuperação do analito. A eluição (para os cátions fracos) ocorre com solução de eluição com o pH duas unidades abaixo do pKa do analito, por neutralização. No sorbente de troca catiônica com ligação a grupos de ácidos carboxílicos alifáticos, a troca iônica é fraca pois o pKa do grupo funcional é de 4,8. Esse tipo de coluna é usada para isolar e recuperar cátions fortes ou fracos, já que o grupo funcional pode ser neutralizado ao utilizar uma solução de pH duas unidades abaixo do pKa pode ser utilizado para eluição neste tipo de coluna (SUPELCO, 1998).

Neste trabalho, foram utilizadas colunas que são tanto de fase reversa como de troca iônica, sendo uma primeira de sorbente de modo misto, troca catiônica forte e fase reversa para bases (Oasis MCX), uma segunda de sorbente de modo misto, troca aniônica forte e fase reversa para ácidos (Oasis MAX) e uma terceira coluna de sorbente de fase reversa para todos os componentes, com equilíbrio hidrofílico-lipofílico (Oasis HLB). Todas as colunas são provenientes da Waters Corp.

## 1.5 CULTURA DA MACIEIRA

A macieira (*Malus x domestica* Borkh.) é uma planta perene de clima temperado pertencente à tribo *Pyreae*, da família das *Rosaceae*, dentro da ordem das *Rosales* (CANTINO *et al.*, 2007). Sua origem é indicada na região montanhosa do Casaquistão, Quirguistão e noroeste da China (CORNILLE *et al.*, 2014). Baseado neste centro de origem, com uma alta variação de temperaturas entre as estações do ano e invernos rigorosos, a macieira apresenta fases de crescimento e dormência bem desenvolvidos e regulados pelo frio (LABUSCHAGNÉ *et al.*, 2002). Para superar a dormência, é necessário o acúmulo de horas de frio ou de resfriamento (CH, do inglês, *chilling hours*), que corresponde ao tempo (em horas) em que a temperatura está menor ou igual a 7,2°C (ou 45°F; CHANDLER, 1937).

Do ponto de vista de produtividade, a macieira é a quarta espécie frutífera no mundo, com uma área mundial plantada de 84.630.275 hectares (ha) em 2014, segundo dados da *Food and Agriculture Organization of the United Nations* (FAO). O Brasil está numa posição de destaque, com uma produção de 1.378.617 toneladas (ton). O Brasil apresenta uma taxa de rendimento de 37,22 ton/ha, enquanto que a média mundial foi de 16,75 ton/ha em 2014 (FAO). As principais cultivares comerciais no Brasil são Gala, Fuji e clones de seus mutantes somáticos (FIORAVANÇO *et al.*, 2016). Para este trabalho, foram utilizadas as cultivares Gala Standard e Castel Gala. 'Castel' é um mutante natural da 'Gala' que requer 300 CH para superar a dormência, enquanto 'Gala' necessita de 600 CH para o mesmo (ANZANELLO *et al.*, 2014). Na Figura 1 está melhor ilustrada a diferença entre estas cultivares a campo, quando 'Castel' já superou a dormência e está em estágio de frutificação, mas 'Gala' ainda encontra-se dormente.

Porta-enxertos de macieiras são utilizados para facilitar o manejo, acelerar o crescimento e antecipar a fase juvenil, proteger de pragas e aumentar o rendimento da produção de maçãs (HOOIJDONK *et al.*, 2011; TWOROSKI & FAZIO, 2011, 2015). Uma vez que há uma diversidade de porta-enxertos, com diversos tipos de resistências bióticas e abióticas, a escolha de qual será utilizado é dada principalmente pelo vigor, sendo os porta-

enxertos ananizantes os preferidos por facilitar as práticas de manejo e a gestão do pomar (HOOIJDONK *et al.*, 2011). Apesar de seu uso fundamental e antigo, pouco se sabe sobre o resultado bioquímico dos porta-enxertos sobre as copas, sendo a caracterização destas plantas baseadas na observação fisiológica dos seus efeitos sobre a cultivar comercial enxertada. Marubakaido (*Malus prunifolia*) é um porta-enxerto originário do Japão e que apresenta grande vigor de crescimento assim como um grande sistema radicular, enquanto M9 (*Malus pumilla*) é um porta-enxerto ananizante que foi desenvolvido no Reino Unido e reprime o crescimento da cultivar enxertada principalmente por transporte ineficiente de hormônios e de carbono causado pelo seu sistema vascular desorganizado (ALVAREZ *et al.*, 1989; HOOIJDONK *et al.*, 2011; TWORKOSKI & FAZIO, 2015, 2016). Além disso, M9 é descrito como um porta-enxerto que atrasa a brotação e, também, como problemático na distribuição de hormônios pelo sistema vascular, em especial as auxinas dos ápices e consequente indução do crescimento de gemas laterais (TWORKOSKI & FAZIO, 2015). A combinação de Maruba com M9 é a mais usada comercialmente pelo fato de proporcionar um tamanho suficiente de árvore para o manejo de poda e colheita, boa resistência às pragas, com produção de frutos grandes e de aparência avermelhada, altamente desejado pelos consumidores (FIORAVANÇO *et al.*, 2016; TWORKOSKI & FAZIO, 2016). Esta combinação de Maruba e M9 é realizada com dupla enxertia: no primeiro ano, há a enxertia do M9 na raiz de Maruba; após um ciclo de crescimento, há a enxertia da cultivar de interesse no M9, formando assim um sistema com 3 genótipos.

Apesar de alguns avanços no entendimento do funcionamento da dormência, ainda há muitas carências sobre questões básicas da modulação do estado de dormência, assim como as influências que a dormência de gemas apresenta por fatores externos, tais como porta-enxertos ou ondas de calor durante o período de acúmulo de frio. Portanto, neste trabalho de mestrado, foram desenvolvidas análises para se obter um avanço de conhecimento sobre essas lacunas, abrindo novas possibilidades para o entendimento da dormência de gemas e para a seleção/indicação de possíveis alvos para ferramentas biotecnológicas de aprimoramento da agricultura.

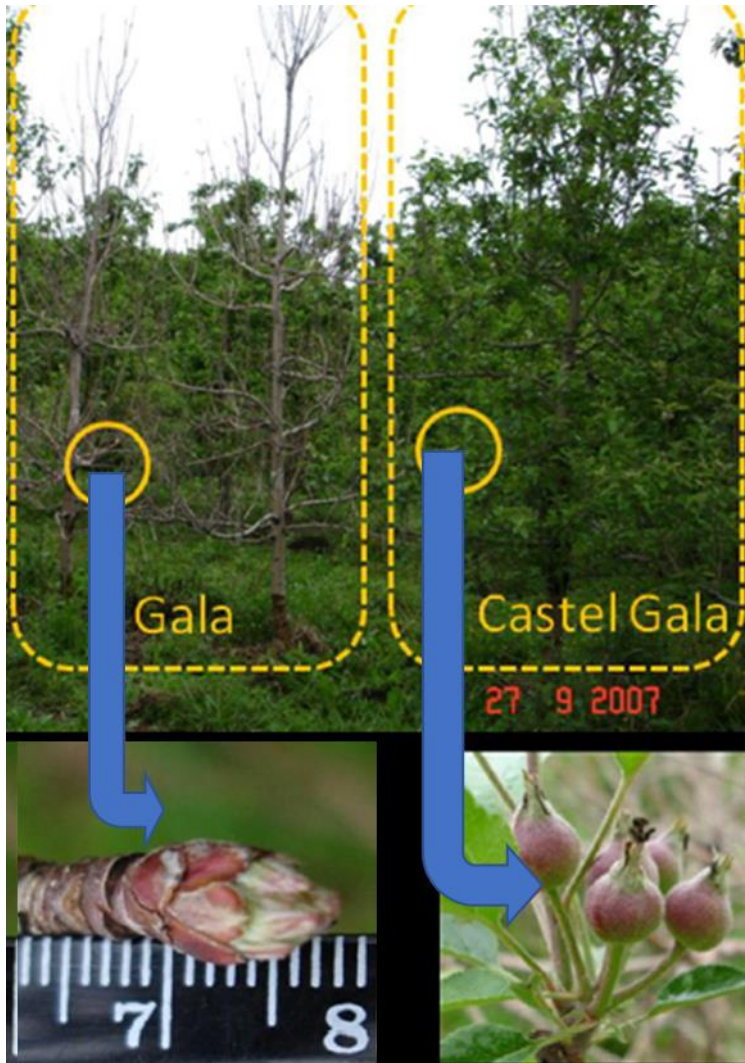


Figura 1: Contraste entre os estados fenológicos de 'Castel' e 'Gala' em 2007. 'Castel' já apresentava frutificação enquanto 'Gala' ainda estava dormente, com eventuais gemas em 'ponta verde'. Imagem retirado de SANTOS *et al.* (2011).



## 2 OBJETIVOS

### 2.1 OBJETIVO GERAL

Utilizando UPLC-ESI-MS/MS, caracterizar o balanço hormonal de gemas dormentes das cultivares de maçã com contrastes de requerimento de frio enxertadas em diferentes porta-enxertos e amostradas em diversos pontos do inverno.

### 2.2 OBJETIVOS ESPECÍFICOS

- a. Desenvolver um método para extrair, purificar e detectar/quantificar diversos hormônios em gemas dormentes de macieira;
- b. Quantificação hormonal das gemas dormentes de macieiras 'Castel' e 'Gala';
- c. Caracterização da influência dos porta-enxertos na modulação hormonal das gemas dormentes;

Os resultados dos estudos relativos ao objetivo específico **a** foram agrupados na forma de artigo a ser submetido para publicação e estão apresentados no **Capítulo I**. O conjunto de resultados relativos aos objetivos **b** e **c** está reunido no **Capítulo II** sob a forma de manuscrito.

### **3 CAPÍTULO I**

**A protocol for hormone extraction and quantification from complex lignified plant bud tissues.**

*Manuscrito a ser submetido ao periódico 'Journal of Chromatography A' (FI: 3,98)*

## **Title**

A protocol for hormone extraction and quantification from complex lignified plant bud tissues.

## **Authors**

Julio de Andrade Garighan<sup>1,2</sup>, Daniel Antunes Souza<sup>2</sup>, Giancarlo Pasquali<sup>1</sup>, Luís Fernando Revers<sup>1,2,§</sup>, Henrique Pessoa dos Santos<sup>2,§</sup>

<sup>1</sup>Graduate Program in Cellular and Molecular Biology, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91501-970, Brazil

<sup>2</sup>Embrapa Uva e Vinho, Bento Gonçalves, RS, 95701-008, Brazil

§Corresponding authors

Dr. Henrique Pessoa dos Santos

Laboratory of Plant Physiology, Embrapa Uva e Vinho, Rua Livramento, 515, P.O. Box 130, 95701-008, Bento Gonçalves, RS, Brazil

**Tel:** +55 54 3455 8126

**Fax:** +55 54 3451 2792

**E-mail:** henrique.p.santos@embrapa.br

Dr. Luís Fernando Revers

Laboratory of Plant Molecular Genetics, Embrapa Uva e Vinho, Rua Livramento, 515, P.O. Box 130, CEP: 95701-008, Bento Gonçalves, RS, Brazil

**Tel:** +55 54 3455 8039

**Fax:** +55 54 3451 2792

**E-mail:** luis.revers@embrapa.br

## Abstract

It is well established that phytohormones play a pivotal role in several physiological processes during plant life cycles, although this knowledge is more qualitative than quantitative. Phytohormones quantification is essential to elucidate the cause of a phenomenon in any physiological process, requiring a universal protocol for quantification. Current methods to extract hormones are not suitable to lignified or dormant buds due to their complex composition and extremely strong matrix effect. These samples are rich in phenols, pigments and subjected to high levels of oxidation that challenges any quantification approach. Matrix effect is a major problem in the analysis of complex samples, and protocols that can overcome this issue are limited and laborious. Therefore, the availability of a sensitive and rapid method that overcomes matrix effect in complex samples to simultaneously quantify multiple classes of hormones is necessary. Given that these analytes are present in very low concentrations, multi-class quantifications need a sensitive instrument and a careful handling during sample preparation. In this work, we developed an extraction method that allowed us to quantify seven different phytohormones in a more sensitive, precise and fast way, besides being able to overcome matrix interferences in lignified or dormant tissues. This method allowed the correct recovery of hormones (104%: spiked standard plus endogenous) while current protocols allow the recovery of less than 10%. This method is a secure way for routinely perform hormone quantification, and to allow the expansion of knowledge especially in the metabolism of plant dormant organs.

## Keywords

Matrix effect, UPLC/MS-MS, dormant organs, SPE cartridge, complex samples

## Highlights

- A new method for phytohormone extraction, purification and quantification is presented
- The presented method allows the extraction and quantification of phytohormones from lignified and/or dormant tissues that are otherwise hard to handle with nowadays methods
- The method is faster, more sensitive and precise than others presented in literature

## Abbreviations

ABA - Abscisic acid; BR - brassinosteroids; CK - cytokinin; ETHY – ethylene; GA<sub>3</sub> - Gibberellic acid 3; GA<sub>4</sub> - Gibberellic acid 4; IAA - Indole-3-acetic acid; JA - Jasmonic acid; KAR – karrikins; LOD – limit of detection; LOQ – limit of quantification; LLE - liquid-liquid extraction; ME - matrix effect; MRM - multiple reaction monitoring; NO - nitric oxide; PVPP – Polyvinylpyrrolidone; SA - Salicylic acid; SL – strigolactones; SPE - solid phase extraction; PA - polyamines; PPT - protein precipitation; UPLC-ESI-MS/MS - Ultra performance liquid chromatography – electrospray ionization – tandem mass spectrometry; Z – Zeatin;

## 1 Introduction

Phytohormones are low abundant molecules that regulate plant metabolism and development [1]. Their minimum quantities trigger basic developmental processes such as cell division, enlargement and differentiation, organ formation, seed dormancy and germination, leaf and organ senescence and abscission [2]. The “classical” first discovered plant hormones are auxins (IAA), gibberellic acids (GA), abscisic acid (ABA), cytokinins (CKs), ethylene (ETHY) and jasmonates (JA) [1,3–5]. Recently, several other plant growth regulators were discovered, such as salicylates (SA), brassinosteroids (BR), polyamines (PA), strigolactones (SL), nitric oxide (NO), karrikins (KAR) and diverse plant peptide hormones [1,6,7]. Plant hormones usually have distinct source and target tissues, hence ensuring long-range communication at the whole-plant level. Plants rely on various hormone distribution mechanisms depending on the distance and the direction of the transport [8]. Their regulation is a complex trait based on a regulatory network with mutual influences on signaling and metabolic systems, retro control and/or control of other hormones [6]. Their relationships define the type of response and its intensity and depend on local and moment that this influence occurs [2]. These interactions were already proved in growth regulation between the balance dynamics of IAA and GAs [2,9]; apical dominance between CKs, IAA and ABA [4,10]; stress responses with ETHY, ABA and GAs [11,12]; and pathogen responses involving SA, JA, and IAA [12]. In all these physiological processes, the relative amount of hormones is accepted as the real regulator of the hormone action rather than their absolute levels [1,6]. In this context, a global hormonal analysis is much more informative instead of a single hormone quantification to evaluate hormone-regulated physiological or to answer developmental biological questions.

Ultra performance liquid chromatography – electrospray ionization – tandem mass spectrometry (UPLC-ESI-MS/MS) is the gold standard analysis technique for tracing analytes with sensitivity, selectivity, and specificity [13–15]. This technique is based on liquid chromatography (to separate analytes in a rank of chemical affinity with the liquid chromatography column) coupled to a mass spectrometry before the ion detector. The transition from liquid medium to

an ionic spray occurs in the ESI, which vaporizes and ionizes the analytes. This system simultaneously considers some characteristics of the target analytes (e.g. retention time, ionization properties and mass transitions) that may ensure the separation, identification and quantification of these in complex mixtures [14].

One of the critical steps of the UPLC-ESI-MS/MS technique is the ionization process, given that a combined effect of all components in the sample, rather than the analyte, alters the measurement of a given molecule, causing problems in the correct ionization and quantification in MS [13,16]. Usually, the ionization interference is due specifically to LC co-eluting substances, enhancing or decreasing signal intensity during sample quantification [17,18] and affecting the reproducibility and accuracy of the assay. [16] This issue is called Matrix Effect (ME) [13,14,19]. Although a great number of approaches were developed aiming to eliminate the ME, only the mitigation or correction of its interference were achieved [15,18, 20, 21]. The most common approach is the utilization of stable isotope-labeled internal standards, usually using deuterated standards [17]. This correction relies on spiking a known amount of a standard directly in the sample (for labeled standards) or in a sample duplicate (for not labeled standards) in the beginning of the extraction step [21]. This procedure allows the measurement of the recovery efficiency, which can be used to calibrate the final quantification result [15], but it still limited to a detectable analyte, not covering false negatives. Although other methods to correct the ME were proposed, such as changes in liquid chromatography conditions [17,18] or ionization source or polarity [14,22,23], their results are often limited. In this context, a main strategy is the employment of better sample purification methods [24]. Several types of purification were designed such as protein precipitation (PPT), liquid–liquid extraction (LLE) and solid phase extraction (SPE), those may be used in combination with precipitation steps and/or antioxidants in order to prevent analyte degradation and false negatives [18]. Most of these methods are specialized for a group of hormones; others to one specific class of growth regulators; and some for hormones in general. These techniques rendered some improvement in ME avoidance, allowing the separation of the analyte

from the co-extracted matrix, and enabling the visualization of ME affected analytes [20]. However, ME remains limiting and interfering in quantitative analysis, requiring more efforts for its avoidance.

The requirements for a better purification method increase with the complexity of the sample matrix. However, available protocols were mostly developed for vegetative tissues, which usually are easier to be handled, especially in *Arabidopsis thaliana* [25]. Conversely, dormant apical buds from apple trees are samples subjected to high oxidation, and are rich in phenols and pigments, offering challenges to any quantification approach [26]. This type of hard/complex sample is difficult to handle with usual protocols, requiring especial manipulation. Lignified, dormant or highly susceptible structures (to oxidation) are becoming more studied and this class of new samples needs a specific and efficient method to allow the extraction, purification and quantification of phytohormones.

In the present work, a multi-hormonal – ABA, GA<sub>3</sub>, GA<sub>4</sub>, IAA, JA, SA and Z – extraction protocol for lignified and complex samples such as apple and grapevine dormant buds, flowers and lignified tissues from bergamot, pear, rose, and seeds from *Araucaria* is described. Variation in extraction, purification and quantification steps were tested and modified until achieving a method that best suited samples. These types of samples were not completely explored by existent methods and here we present a functional methodology to quantify even phytohormones that occur in minute amounts, such as IAA, in dormant or lignified tissues.

## **2 Material and Methods**

### *2.1 Extraction Method*

#### *2.1.1 Plant Material*

Terminal dormant buds from apple trees (*Malus x domestica* Borkh.) were used. Buds were fully closed (stage A according to the Fleckinger scale [27]) from six 'Castel Gala' and two 'Gala Standard' apple trees grafted on

Marubakaido rootstocks. Samples were harvested in May, June, July and August of 2008 in a commercial orchard at Monte Castelo, SC, Brazil (26°37'39"S, 50°14'7.73"W and 791 m altitude), immediately frozen in liquid nitrogen in the field and stored at -80°C until use, as previously described by Falavigna *et al.* [28]. Buds from 'Gala Standard' and 'Castel Gala' were mixed before the analysis to generate a more complex sample.

### 2.1.2 Extraction Protocol

Samples of intact buds were ground in a cryogenic impact grinder (Freezer/Mill 6870, SPEX SamplePrep Co., USA) with liquid nitrogen, obtaining a fine powder that was kept at -20°C until use. Four mL of cold (-20°C) extraction solution was added to the fine powder and vigorously mixed in a vortex for 20 s. Different extraction solutions were tested and are detailed in section 2.1.2.1 with adjuvant/antioxidant assay detailed in section 2.1.2.2. Different weights and ratios of extract solution to sample mass – section 2.1.2.3 – were tested. Samples were incubated at -20°C for 3 h and then transferred to an ultrasound bath (Soniclean 2PS, Sanders, Brazil) with 40 kHz frequency for 25 min at 4°C, followed by a centrifugation step of 1,750 x g for 30 min at 4 °C (Micro 17TR, Hanil Science Industrial, South Korea). The supernatant was stored, and the precipitates were re-extracted two-times with 3 mL of extraction solution each time, being suspended by vortex again each time. During the second extraction, samples were kept for 6 h at -20°C before sonication. Incubation was of 12 h in the third extraction. After the last centrifugation step, supernatants were combined and then purified according to section 2.2.2.

#### 2.1.2.1 Extraction Solution

Four extraction solutions were tested aiming to determine the most stable and efficient one: ES1 (acetonitrile:water:formic acid, 80:19:1, v/v) [29]; ES2 (chloroform:methanol:water:formic acid, 25:60:10:5, v/v) [30]; ES3 (methanol:water:isopropanol:formic acid, 50:20:20:10, v/v) and ES4 (methanol:water:formic acid, 75:20:5, v/v) [3]. All solutions contained 2 mM citric acid as antioxidant. In order to guarantee the evaluation of the extraction efficiency of each solution on endogenous hormones, the addition of exogenous standards was not used at this assay. Methanol, acetonitrile, isopropanol and



formic acid were purchased from Sigma-Aldrich, USA; chloroform was from J.T. Baker, USA. Water was purified with a Milli-Q equipment (MerckMillipore, USA).

#### 2.1.2.2 Antioxidant assay

In order to prevent a possible ME presence resulting from oxidation and decarboxylation losses such as the decarboxylation of IAA by SA [31], citric acid, ascorbic acid and dithiothreitol (DTT) were tested in the concentrations of 0.1 mM, 1 mM, 2 mM and 4 mM. These antioxidants were applied in the extraction solution before the addition of samples, and the recoveries were analyzed according to literature's Recovery Efficiency definition [17].

#### 2.1.2.3 Proportion Between Sample and Extraction Solution

Five different proportions were tested to evaluate the best ratio between sample and extraction solution. Using 10 mL of extraction solution as a fixed volume, the weight of the samples varied in 200 mg, 500 mg, 1,000 mg, 2,000 mg and 3,000 mg, rendering ratios of 20 mg/mL, 50 mg/mL, 100 mg/mL, 200 mg/mL and 300 mg/mL, respectively. Samples were extracted by ES4 with 2 mM citric acid and purification on SPE Oasis MCX, since this was the best condition so far.

### 2.2 Purification method

Complex samples usually present high pigmentation and interfering components, being necessary a purification step following the extraction [32,33]. SPE was chosen due to fast, cheap and ease use, in addition to its widespread employment. Several purification methods were tested such as the use of several different SPE cartridge protocols and combinations (section 2.2.2), dilutions and drying method (section 2.2.1) as follows.

The supernatant coming from the extraction was usually dried in a vacuum concentrator (6,700 g at 40°C under vacuum) and suspended in 2 mL of 1 M formic acid. The suspended samples were applied onto SPE cartridge (assay detailed in section 2.2.2). Focusing on optimizing the purification step,

variations in SPE DSC-18 combination with drying step, resuspension modes and types of ionic SPE were tested, as detailed in the next sections.

### 2.2.1 Methanol Content Dilution

Given that several phytohormones are soluble in alcohol [3,34], a solvent present in many extraction buffers, its elimination in further steps are often required. Considering that the drying step takes time, the possibility of dilution of alcoholic contents before applying the extract onto the Oasis SPE (Waters Co., Milford, MA, USA) cartridges was tested rather than drying the alcohol out. Sample was extracted using the ES4 solution [3]. After extraction, a sample totally dried in a vacuum concentrator (6,700 g at 40 °C, under vacuum) and reconstituted in 1 M formic acid was used as control, and for the others was added 1 M formic acid in several volumes with the purpose of evaluating three dilution concentrations: from 75% to 15%, to 7.5%, or to 3% alcoholic concentration.

### 2.2.2 SPE Cartridges

Three SPE cartridges, Oasis MCX, MAX and HLB (Waters Co., Milford, MA, USA) were selected due to their wider pH range and ion exchange capability. Each Oasis cartridge was initially prepared as described for MCX [32], MAX [34], and HLB [35]. All testing procedures used the ES4 extraction solution [3], once this was the best condition so far. The first analysis of the Oasis cartridge were carried with hormone standards spiked in the extraction solution immediately after the sample, as described in section 2.3.1.

#### 2.2.2.1 Oasis Max

Oasis MAX cartridges (Waters Co., Milford, MA, USA) were initially wetted with 5 mL methanol followed by 5 mL of 1 M formic acid. Extracted supernatants were previously dried in a vacuum concentrator (6,700 g at 40°C, under vacuum) and suspended in 3 mL of 1 M formic acid and then loaded onto

the Oasis MAX cartridge. The cartridges were washed with 5 mL of 10 mM potassium phosphate buffer (pH 7) followed by 5 mL water wash. Hormones were eluted first with 5 mL methanol, with one additional elution of 5 mL methanol containing 1% formic acid. The obtained eluates were dried, reconstituted in 75  $\mu$ L of methanol and then filtered through a 0.22- $\mu$ m PVDF filter for LC–ESI-MS/MS analysis, as described in [34].

Aiming to reduce the presence of hydrophobic compounds in the extracts, as described in [33], an assay with Discovery<sup>®</sup> DSC-18 SPE (Sigma-Aldrich, Saint Louis, USA) cartridge was performed in combination with Oasis MAX, before drying the sample, as follows (summarized in Table 1):

1 - Use of DSC-18 SPE cartridge and Oasis MAX with acid wash (3 mL 1 M formic acid) identified as ACA (named as ACA due to anionic cartridge, DSC-18 and acid wash); 2 - use of DSC-18 SPE cartridge and Oasis MAX with basic wash (3 mL 3% ammonium hydroxide in methanol) identified as ACB; 3 - absence of DSC-18 SPE cartridge use, Oasis MAX with acid wash (3 mL 1 M formic acid) identified as ASA; 4 - absence of DSC-18 SPE cartridge use, Oasis MAX with basic wash (3 mL 3% ammonium hydroxide in methanol) identified as ASB.

*Table 1. Summary of assays combining different washes in Oasis MAX and use of DSC-18 before Oasis MAX.*

| MAX SPE settings | Acid wash | Basic wash |
|------------------|-----------|------------|
| With DSC-18      | ACA       | ACB        |
| Without DSC-18   | ASA       | ASB        |

#### 2.2.2.2 Oasis HLB

The SPE cartridge was previously wetted with 5 mL methanol followed by conditioning it with 5 mL methanol:water:acetic acid (10:89:1, v/v). The extracts were dried out and dissolved in 3 mL of methanol:acetic acid (99:1, v/v) and

then mixed with 2 mL of 1% acetic acid. Samples were loaded onto Oasis cartridges and washed with 5 mL methanol:water:acetic acid (10:89:1, v/v). Then hormones were eluted by 5 mL methanol:water:acetic acid (80:19:1, v/v). The obtained eluates were dried out, reconstituted in 75  $\mu$ L of methanol and then filtered through a 0.22- $\mu$ m PTFE filter for LC–ESI-MS/MS analysis, according to [35].

### 2.2.2.3 Oasis MCX

The SPE Oasis MCX cartridge (Waters Co., Milford, MA, USA) was wetted with 5 mL methanol followed by conditioning it with 5 mL 1 M formic acid. Thereafter, samples were applied (after dried and reconstituted in 3 mL 1 M formic acid) and the cartridge was washed with 3 mL 1 M formic acid and eluted with 5 mL methanol (Elute 1) followed by 5 mL 5% ammonium hydroxide in methanol (Elute 2) as in [32]. Samples presented a dark color in Elute 2, which may interfere or even damage the MS/MS analysis. Aiming to reduce the color, a reduction in ammonium hydroxide was tested from 5% to 3% with equal hormone recovery, however, the Elute 2 remained still colored. Aiming to solve this issue, it was tested the Discovery<sup>®</sup> DSC-18 SPE (Sigma-Aldrich, Saint Louis, USA) as described in [33], in four combinations with Oasis MCX (summarized in Table 2): (1) in the absence of C18, with the normal elution on Elution 2 identified as CSN (Cationic, DSC-18 less, NH<sub>4</sub>OH 3%); (2) with the use of DSC-18 and normal Elution 2, identified as CCN (Cationic, DSC-18, NH<sub>4</sub>OH 3%); (3) DSC-18 sample purification and serial elution of 3% ammonium hydroxyde in methanol from 0 to 100% (in steps of 20%), named CCL (Cationic, DSC-18, Long elution); (4) same serial elution of 3% ammonium hydroxyde in methanol without DSC-18, named CSL (Cationic, DSC-18 Less, Long elution); the serial organic content proportions tested were 0%, 20%, 40%, 60%, 80% and 100% methanol in water in Elution 2 in steps of 1.5 mL.

*Table 2. Summarize of the assay combining different elutions in Oasis MCX and the use of DSC-18 before Oasis MCX.*

|                | Direct Elute 2                                    | Serial Elute 2   |
|----------------|---|--|
| MCX settings   | <b>3% ammonium hydroxyde in<br/>100% methanol</b> | <b>3% ammonium hydroxyde in<br/>methanol (0 to 100%, steps of 20%)</b> |
| With DSC-18    | CCN   | CCL  |
| Without DSC-18 | CSN   | CSL  |

In order to guarantee a greater recovery of the hormones without the presence of these interfering compounds, an assay was performed to diminish the concentration of ammonium hydroxide. It was tested different ammonium hydroxide concentrations, with serial elutions from 0.004, 0.04, 0.4, 4 and 40 mM concentration in methanol:water (4:1, v:v, defined in section 3.2.2.2) for the Elution 2 step in Oasis MCX. A final wash composed of 3% ammonium hydroxide in methanol (0.8 M) was used as strong hormonal cartridge release to verify hormone loss in the SPE cartridge. The obtained eluates were dried out, reconstituted and filtered as previously described before LC-ESI-MS/MS analysis.

### 2.2.3 Flow Through

In order to evaluate the influence of flow speed on the purification performance of SPE cartridges, different volumes per time were evaluated: 0.1, 0.3, 5 and 10 mL/min based on [32,36,37]. The assay was performed in Oasis MCX SPE cartridges and flows were controlled by a SPE vacuum manifold (Visiprep™, 12-port model).

### 2.2.4 Polyvinylpolypyrrolidone (PVPP) assay

PVPP was tested as a sample pigment cleaner, using 1,7% PVPP, adapted from [38]. Vortexing (30 s) was applied to samples and they were kept in -20°C for 30 min. The vortexing and cold incubation were repeated once in the same conditions. Samples were then centrifuged (10,000 g for 45 min at 4°C) and supernatants were collected. In order to identify the best time to perform the PVPP cleaning and if this procedure promoted some impact on the amount of each hormone, the following assays were employed: (1) extraction with ES4, drying and suspension in water, PVPP purification and use of Oasis MCX, followed to LC-MS analysis; (2) extraction with ES4, PVPP, drying and suspension in water, followed by Oasis MCX and LC-MS analysis; (3) extraction with ES4, drying and suspension in water, PVPP, Oasis MCX, PVPP, LC-MS; (4) extraction with ES4, drying and suspension in water, Oasis MCX, PVPP, LC-MS; and (5) extraction with ES4, drying and suspension in water, PVPP and LC-MS. All variations were tested with and without standards.

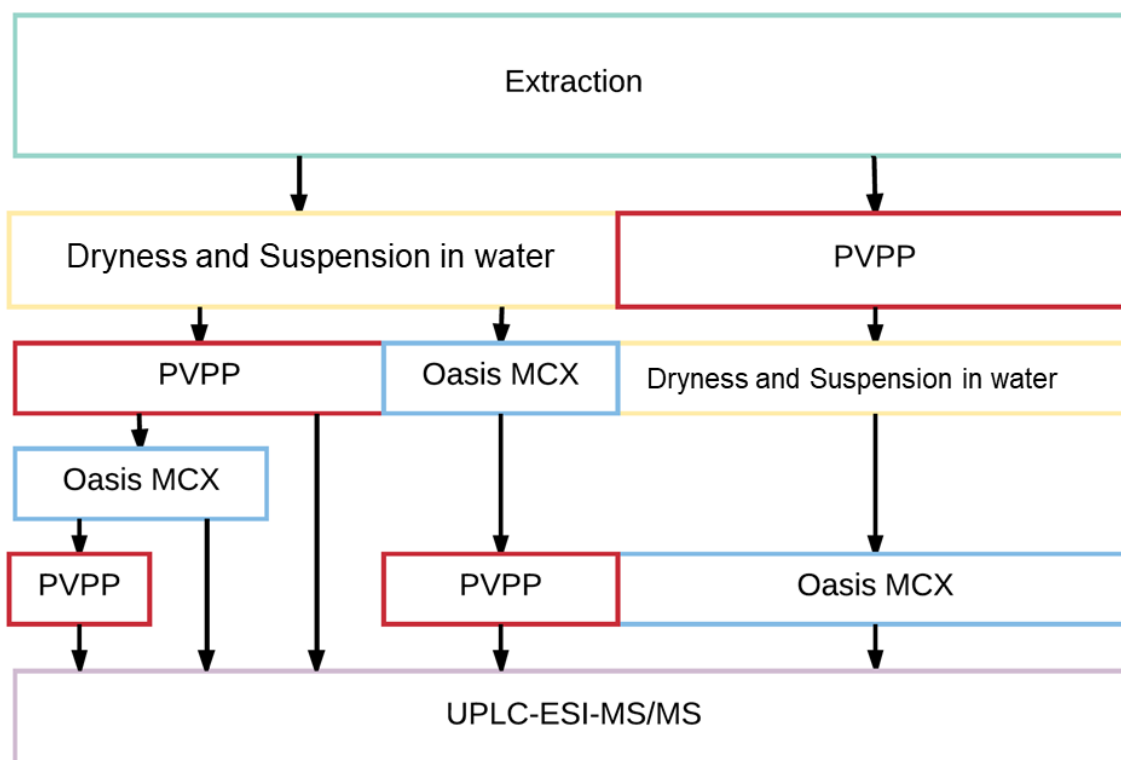


Figure 1. Summary of PVPP combinations with Oasis MCX from extraction to quantification.

## 2.3 Detection/Quantification Method

Hormone quantification was performed in a UPLC-ESI-MS/MS system equipped with an Acquity UPLC™ (Waters Co.) quaternary pump with an auto-sampler, coupled into a Waters Xevo™ triple quadrupole mass spectrometer system (MS/MS) employing an ESI interface. In this step, hormone standard solutions were used as references in the chromatographic and detection adjustments. After basic settings, samples (with and without hormonal standard) were used to fine adjustments of UPLC-ESI-MS/MS parameters such as retention time, gradient modulation and pH of LC solutions.

### 2.3.1 Standard Curves and Hormonal Standard Spike

Standards for ABA (A1049), SA (S5922), GA3 (G7645), GA4 (G7276), JA (J2500) IAA (I2886) and Z (Z0164), all from Sigma-Aldrich, were dissolved in methanol (1 mg/mL). Standard curves were prepared in three replicates with concentrations of 5, 10, 50, 100, 250, 500, 1,000, and 1,500 ng/mL. Their quantification was performed in triplicate in the UPLC-MS/MS. For quantification, the limits of detection (signal/noise, S/N>3) and the quantification (S/N>10) were respected using the TargetLynx™ software (Waters, USA). To determine the recovery efficiency and matrix effect (ME) (section 2.4), 4 µL of 5 µg/mL of all standards were spiked in each sample at the beginning of the extraction.

### 2.3.2 UPLC-ESI-MS/MS Method

A set of Acquity BEH C18 VanGuard (Waters, USA) pre-column (2.1 × 5 mm, 1.7 µm) and column (2.1 × 50 mm, 1.7 µm) was applied for the chromatographic separation. The system was adjusted to maintain 4°C and 40°C in the sample and column compartments, respectively. The detection step was adjusted to the following conditions: capillary voltage, 2.5 kV; source temperature, 150°C, desolvation temperature, 500°C; desolvation gas flow, 600 L/h; cone gas flow, 100 L/h, collision gas flow 0.5 mL/min. Multiple reaction monitoring (MRM) modes were used to monitor the transitions from the precursor ions to the most

abundant product ions, optimized by direct infusion of each hormone. The MRM transition results of the different phytohormones monitored are in Table 3.

*Table 3: MRM transition and optimal parameters for MS-MS acquisition and each phytohormones.*

|                       | ABA     | GA3     | GA4     | IAA     | JA     | SA     | Z       |
|-----------------------|---------|---------|---------|---------|--------|--------|---------|
| MRM transition (m/z)  | 263>153 | 331>213 | 345>142 | 174>130 | 209>59 | 137>93 | 220>136 |
| Cone voltage (V)      | 40      | 60      | 35      | 30      | 24     | 34     | 30      |
| Collision voltage (V) | 20      | 32      | 23      | 15      | 17     | 18     | 17      |
| Ionization mode (ESI) | Neg     | Neg     | Neg     | Pos     | Neg    | Neg    | Pos     |

In order to improve the chromatographic conditions for separation of the target compounds as well as to reduce the ME during the detection of samples (with hormonal standard spikes), the flow was fixed to 0.45 mL/min, and eluent and gradient variations were performed in the mobile phase (MP), as follows:

MP 1) A: water + formic acid 0.1% / B: acetonitrile + formic acid 0.1%;

MP 2) A: water + formic acid 0.1% / B: acetonitrile:methanol (1:1, v/v) + formic acid 0.1%;

MP 3) A: water + ammonium hydroxide 0.05% / B: acetonitrile + ammonium hydroxide 0.05%.

Within each eluent variation and following a factorial design, three elution gradients (G) were also tested, as described below:

G 1: 1 to 100% B in 2.2 min, keeping 100% B up to 2.8 min and turning to initial condition of 1% B in 2.9 min with end in 3.2 min;

G 2: 0 to 10% B in 2 min and from 10% to 100% in 4.2 min, keeping 100% B up to 4.8 min and turning to initial condition of 1% B in 4.9 min with end in 5.2 min;

G3: 1 to 20% B in 2 min, from 20% to 38% in 4.2 min, from 38% to 100% in 6.5 min, keeping 100% B up to 8.5 min and turning to initial condition of 1% B in 8.6 min with end in 9.5 min.



## 2.4 Matrix Effect (ME)

The extraction, purification and chromatographic separation steps were evaluated for their accuracy, process efficiency and also ME, as described [17]. Samples were spiked with hormonal standards in the beginning of the extraction and analyzed for recovery in detection/quantification step. The ME was considered as an interference when hormone standard recovery was affected more than 10%.

## 2.5 Validation Steps

From the definition of the most promising conditions at each stage of the protocol, some hormonal quantification assays were performed on different plant tissues. These assays aimed to the possibility of using the method in other tissues, as well as the contrast that each matrix effect can exert on the detection capability of the different hormonal classes. Different samples with complexes and/or lignified matrices were chosen, with no previous report of hormonal quantification. Samples presented huge issues to common quantification methods as high pigmentation, dormant and/or lignified tissues, and the presence of large quantities of carbohydrates.

The samples used to test versatility of the method were dormant buds of *Vitis vinifera* L., cv. Pinot Noir harvested during Winter in August 2016; floral buds and leaves from rose (*Rosa × kordesii*), pear (*Pyrus communis* L.), and bergamot (*Citrus bergamia*), harvested in July and August 2016. All plant material was collected in Bento Gonçalves, RS, Brazil (-29°16'35"S, 51°53'44"W, 691m). *Araucaria angustifolia* sprouts were harvested in March, April, May and June 2014 and 2015, in Urubici, SC, Brazil (-28°00'54"S 49°35'31"W, 915m). All samples were immediately frozen in liquid nitrogen in the field and stored at -80°C until use.

## 2.6 Statistical Analysis

All data were analyzed using GraphPad Prism version 6.00 for Windows, GraphPad Software (La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com)). Data were

processed using two-way ANOVA repeated measures followed by Tukey and Bonferroni *post hoc* tests.

### 3 Results and Discussion

Apple dormant buds present several interfering compounds. Due to their physical-chemical characteristics, methods of extraction and quantification nowadays employed were not able to succeed satisfactorily phytohormone measurements in these samples. Therefore, aiming the quantification of multiple phytohormones in this type of lignified and complex organs, some adjustments in extraction, purification and quantification steps became necessary. In the following section, all particularities of assays developed and tested will be specified and discussed in each step of the protocol to identify optimal conditions.

#### 3.1 Extraction Step

##### 3.1.1 Extraction Solution

All four solutions tested as described in section 2.1.2.1 (ES1 methanol-based, ES2 acetonitrile-based, ES3 chloroform-based and ES4 methanol/isopropanol-based) exhibited the same performance in phytohormone extraction, with an average recovery of less than 17 ng of all hormones per gram of sample (Figure 2). However, previous reports showed differences in extraction efficiency among different groups, with preference for some hormonal groups during extraction depending on the solution composition [3]. Previous work reported this type of result with *Rosmarinus officinalis* as an example of complex sample [3], but apple buds seems to be even more complex samples. These contrasting results could be related to other restriction factors rather than just the extraction solution. Several other interfering components may have great influence on the extraction procedure such as oxidative processes that can degrade the analytes and diminish the efficiency of the extraction solution. *R. officinalis* presents a significant group of natural phenolic compounds that act as antioxidants like rosmarinic acid [39], caffeic acid [40] and carnosic acid [41],

which are not described as abundant in apple dormant buds. The presence of natural antioxidants may explain the standard spiked recovery in literature for *R. officinalis* and other oleaginous plants. This lack of variation could be also due to limited efficiency in the method, requiring testing of next variables to overcome the problem.

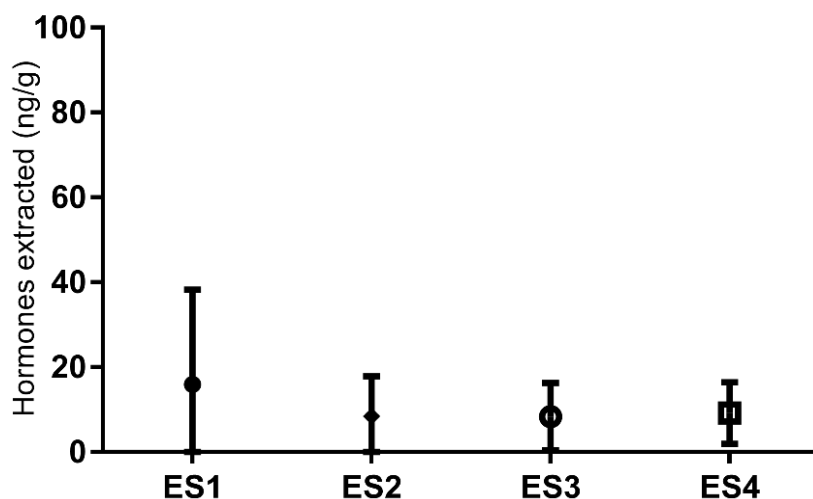


Figure 2 Comparison of extraction efficiency among different Extraction Solutions. Average extraction of all hormones from apple buds extracted with ES1 (acetonitrile-based); ES2 (chloroform-based); ES3 (methanol/isopropanol-based) and ES4 (methanol-based).

### 3.1.2 Antioxidant in Extraction Solution

In a trial to solve the issue of low extraction and recovery efficiency compared with *R. officinalis* [3], assays with antioxidants were performed to discern if the cause of low efficiency was tissue specific (due to oxidation) or method specific (mishandling of samples). Diverse antioxidants in different concentrations were tested. In the overall phytohormone analysis in relation to the type of antioxidant employed during extractions (section 2.1.2.2), there was no difference among ascorbic acid, DTT or citric acid (Figure 3a). In the concentrations tested, there was no difference in the recovery efficiency of phytohormones among antioxidants (Figure 3b). Seeking an explanation for these similarities, we observed that the extraction solutions employed have pH near 2, which disqualify DTT as antioxidant, despite its large use in protein or

RNA extraction [38]. The reducing power of DTT becomes less potent as the pH gets lower than 7, because only the negatively charged thiolate form  $-S^-$  is reactive, while the protonated thiol form  $-SH$  is not [42,43]. Moreover, DTT half-life is only about seven hours under this condition [43,44], preventing long time extractions or the previous preparation of extraction solutions. Farrow & Emery [45] reported that acidic pH creates an environment conducive to phytohormone degradation, especially IAA, limiting up to 16 h-single extraction time [45]. In contrast, other works [31,46] reported that the use of DTT increases IAA protection when germinating rice seeds were extracted with 80% acetone in water with 2.5 mM DTT for 3 h, which differs from conditions described by Farrow & Emery. Since the extraction solutions were not the same as those employed in rice seed extraction, the focus of antioxidant use was to prevent IAA degradation because our conditions were similar to those described by Farrow & Emery [45].

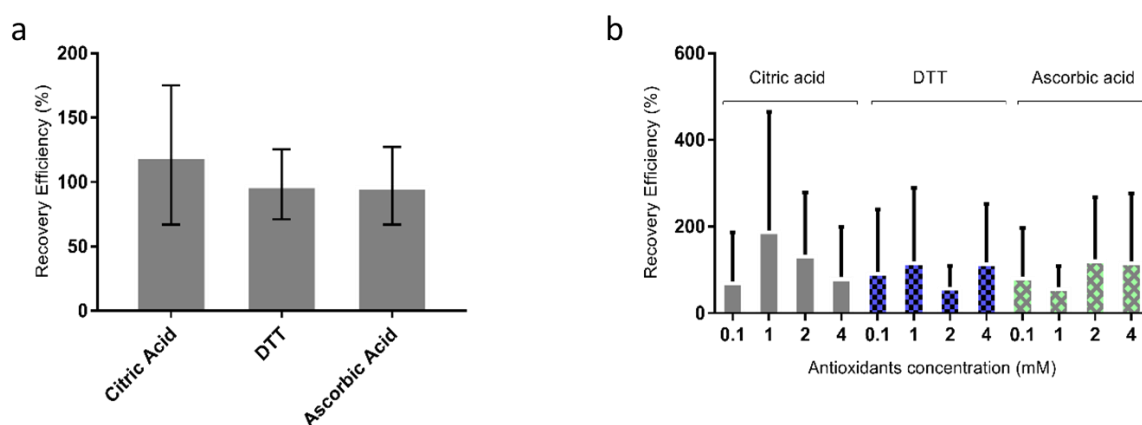


Figure 3 – a) Comparison of standards spiked recovery efficiency among extraction solutions with different antioxidants: citric acid, DTT or ascorbic acid. B) Comparison of standards spiked recovery efficiency among antioxidant in different concentrations (0.1; 1; 2 and 4 mM). bars represent SD.

Considering that usually IAA is present in low concentrations and is rapidly degraded in the presence of high concentrations of SA [31], its protection is an essential issue in the development of a multi-hormone quantification protocol, without disturbing the analysis of the other analytes,

especially in dormant or highly complex organs. IAA relative recovery was measured as IAA efficiency of extraction over other hormones, aiming to identify the minor antioxidant concentration that promoted the greater IAA relative quantification increase (Figure 4). Based on our results, the best relative IAA extraction was achieved when 2 mM citric acid was employed as antioxidant in apple dormant bud samples. This was used as antioxidant condition in the following assays.

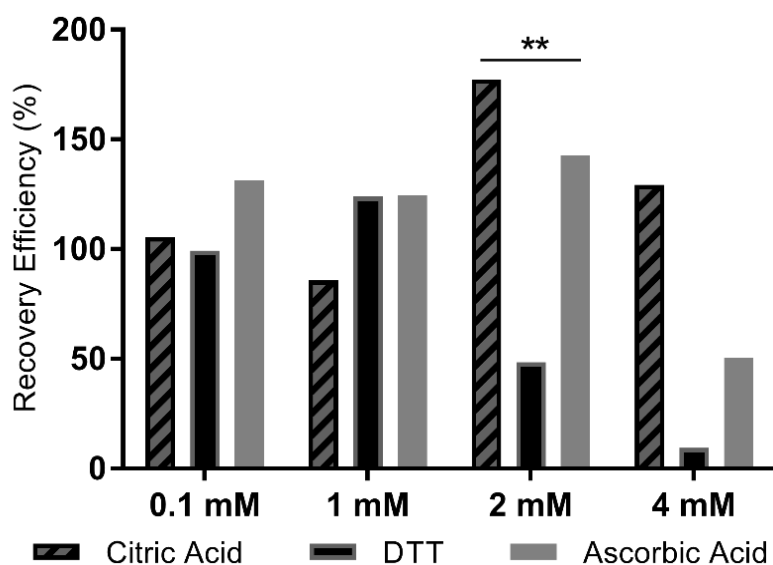


Figure 4 Comparison of relative recovery efficiency of IAA among antioxidants in different concentrations. Relative recovery efficiency of IAA represents the ratio between extraction efficiency of IAA and the mean of other hormones: values greater than 100 means higher protective effect to IAA in comparison to others hormones. Anova followed by Bonferroni test: \*\* p<0.05, others columns are in \*\*\* p<0.01

### 3.1.3 Balance between Sample and Solution

Among the factors that may also impact the efficiency of hormone extraction is the proportion of the sample in relation to the volume of solution. In this assay (section 2.1.2.3), the highest extraction efficiency was observed with 50 mg/mL sample:extraction solution, with the second-best result with 100 mg/mL ratio (Figure 5). The lowest extraction efficiency was obtained for the ratio 200 mg/ml, probably by difficulties during suspension. This was an unexpected result since this was the proportion used in satisfactory results in previous works [45,46]. This can be due to sample type used in those works,

i.e., *A. thaliana* leaves or rice calli that are easier to handle. Another possibility is that the oxidative power of the sample (bud tissues) exceeded the antioxidative power of the extraction solution, or the interfering compounds that coelute with the analytes could mitigate the ionization of target molecules (ME) during the transition between LC and MS [17], since those tissues present natural antioxidants in greater quantities than lignified dormant tissues [39–41]. These make them less visible or even complete invisible to the detector, which explains why 300 mg/mL also showed lower extraction efficiency than the ratio of 50 mg/ml. Therefore, we defined 50 mg/ml (10 ml solution extraction and 500 mg of apple dormant buds) as the best balance between sample mass and extraction solution. Despite the fact that usual plant tissues used in extraction methods are soft and colorless tissues, apple dormant buds and other lignified tissues have a high proportion of phenolic compounds [26], which interfere with the ionization process during analysis. In fact, apical meristem represents approximately 12% of apple dormant bud mass composition, and less than 5 % in grapevine dormant buds (measurements in our lab). Considering only the mass of the apical meristem from 500 mg of dormant buds, this protocol allows the extraction of phytohormones from less than 65 mg (for apple dormant buds) or 25 mg (for grapevine dormant buds), despite of highly interfering compound quantities or highly ME contaminated samples with a low active metabolism.

Following the selection of best type and concentration of antioxidant (citric acid at 2 mM) and the best ratio of sample and extraction solution, focus was on the choice of extraction solution itself. Considering that there were no significant differences in extraction efficiency among solutions tested, an important point of loss could be the purification step (SPE) or the combination of both. The best extraction solution, considering the extraction of undesirable or interfering compounds that may be carried out, should provide lower hormone losses in SPE charge in Flow through (F) and maximum elution on elution phase (E). Despite the equality in quantification on E, extraction solutions performed quite differently on hormone loss in F. ES1 resulted in 80 ng/g hormone loss, ES2 lost 19 ng/g, ES3 lost 4.3 ng/g and ES4 lost 1.6 ng/g (Figure 3). Therefore, although they showed similar elution efficiency, the acetonitrile-based extraction solution (ES1) presented a high hormone loss in the first step

of SPE. It occurred probably because acetonitrile has a higher elution efficiency than methanol [47]. ES4, the methanol-based extraction solution, was selected as the best one since it is widely used [3,32–34], ease to employ and with the lower hormone loss during the Washing step (Figure 6).

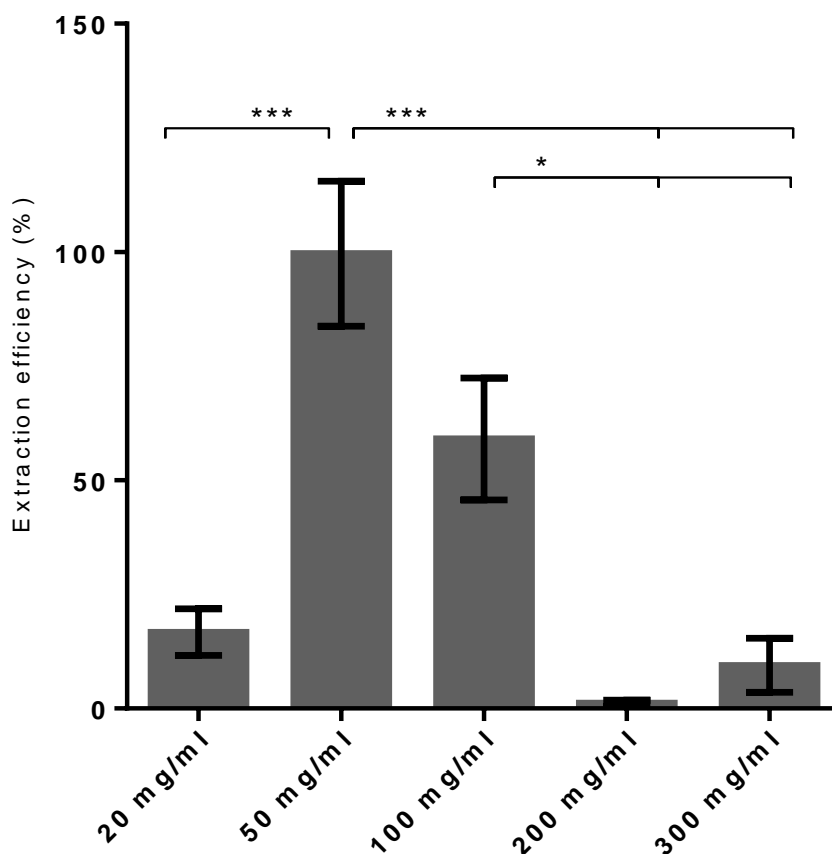


Figure 5 Comparison of extraction efficiency among diverse proportions of sample and extract solution. \*\*\* means  $p < 0.01$  and \* means  $p < 0.1$

#### 3.1.4 Alcoholic drying before SPE

An extract dilution assay was done to avoid the need to dry the sample before elution in SPE columns, in order to save time. In the *Methanol content dilution* assay (section 2.2.1), differences were observed between the Dried Sample Method and the Diluted Sample Method. No significant difference among diluted samples were observed. The Diluted Sample Method showed lower hormone recovery ( $< 30\%$ ) while the method of Dried sample exhibited satisfactory hormonal recovery ( $> 95\%$ ; Figure 7).

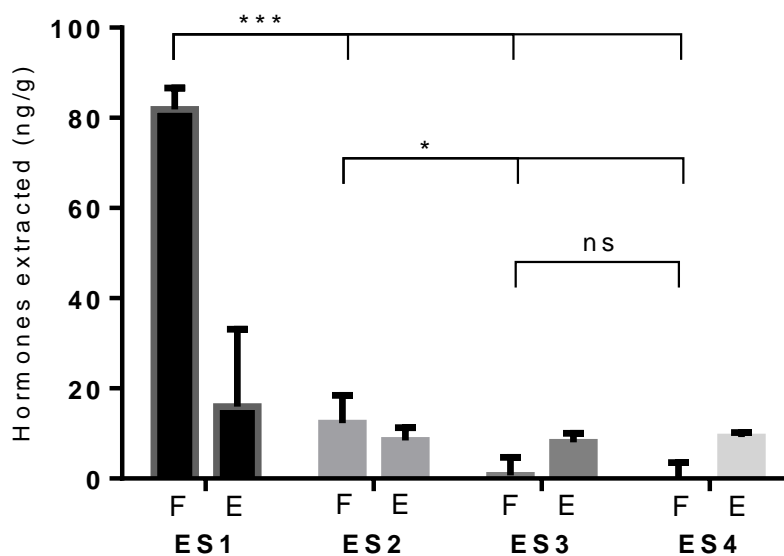


Figure 6 Comparison of extraction efficiency and hormone loss among different extraction solutions: F - hormone loss in Flow through and Wash on SPE; E - hormones eluted correctly. ES1 – acetonitrile:water:formic acid (80:19:1, v:v); ES2 – chloroform:methanol:water:formic acid (25:60:10:5, v:v); ES3 – methanol:water:isopropanol:formic acid (50:20:20:10, v:v) and ES4 – methanol:water:formic acid (75:20:5, v:v). \*\*\* means  $p < 0.01$ ; \* means  $p < 0.1$ ; ns means ‘no significance’

These data allowed us to define that dilution was not the best option for extract preparation before the SPE purification step. Two factors could compromise hormone recovery: (i) hormones are dissolved in methanol even in high dilution, given that the proportion was changed but not the alcoholic quantity. It results in analyte loss no matter the dilution rate that was employed; (ii) the hormones may be fixed in the column and the analytes were eluted by flow, given that the volume after dilution is higher (50 ml for 15%, 100 ml for 7,5% and 250 ml for 3% methanol content) than the one supported by the cartridge (3 ml without elution by flow). Possibly the two events can occur simultaneously, and this issue could be eliminated only with the complete drying of alcoholic content in the sample extract. These data corroborate previous findings [48].



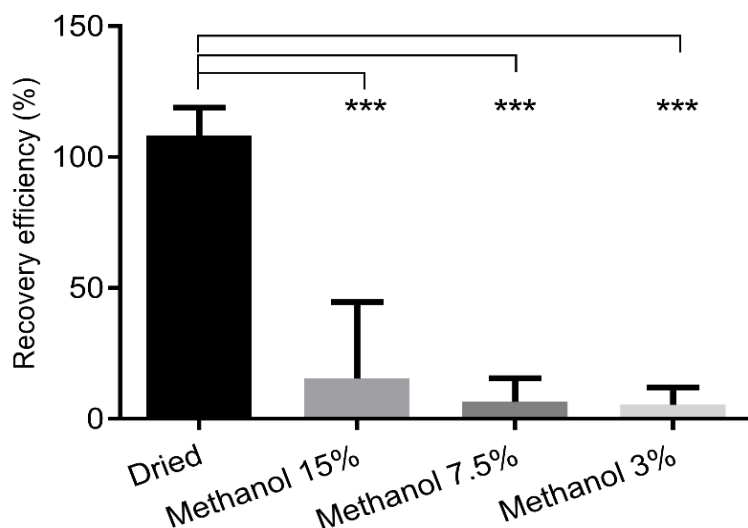


Figure 7 Comparison of hormone recovery efficiency among different dilutions and dryness treatments of alcoholic content. \*\*\* means  $p < 0.01$

After all the assays and modifications evaluated, the extraction step defined can be summarized as follows: powdered samples (500 mg) are added directly in 4 ml of extraction solution (methanol:water:formic acid, 75:20:5, v/v) with 2 mM citric acid at  $-20\text{ }^{\circ}\text{C}$ , agitated by vigorous vortexing (20 s) and kept at  $-20\text{ }^{\circ}\text{C}$  for 3 h. After that, samples are transferred to an ultrasound bath adjusted to 40 kHz frequency for 25 min at  $4\text{ }^{\circ}\text{C}$ , followed by a centrifugation step of  $1,750 \times g$  for 30 min at  $4\text{ }^{\circ}\text{C}$ . The supernatant is stored, and precipitates are re-extracted twice with 3 mL extraction solution each time, being suspended by vortexing again each time. In the second extraction, samples are kept for 6 h at  $-20\text{ }^{\circ}\text{C}$  before sonication and 12 h for the third extraction. After the last centrifugation step, supernatants are combined and totally dried in a vacuum concentrator ( $6,700 \times g$  at  $40\text{ }^{\circ}\text{C}$ , under vacuum) and reconstituted in 1 M formic acid before purification in SPE.

### 3.2 Purification step

The extracts from dormant buds presented a dark red pigmentation, indicative of contamination that was undesirable to use in UPLC-ESI-MS/MS since it could obstruct the system flow and damage the apparatus. In order to

circumvent this issue, a purification step was necessary and Solid Phase Extraction (SPE) was chosen due to its characteristics of being fast, cheap and easy to use, in addition to its widespread application [48,49].

### 3.2.1 SPE cartridges Oasis MCX, HLB and MAX

In the SPE assays, the recovery efficiency of spiked standards was of 0.17% for MCX, 0.03% for MAX and 0.74% for HLB (Table 4). These low recovery rates could be directly related to the matrix type because the solution with spiked standards and the one without the sample presented the same problems but with higher recovery efficiencies, with 6.25% for MCX, 27.18% for MAX and 3.24% for HLB. Even so, they exhibited a low performance in spike recovery, showing that the issue is also related to SPE itself and not only with matrix type.

*Table 4 SPE Recovery efficiency of spiked standards in sample and blank solution.*

| SPE            | MCX    | HLB   | MAX    |
|----------------|--------|-------|--------|
| Sample         |        |       |        |
| Complex matrix | 0.17 % | 0.74% | 0.03%  |
| Blank solution | 6.25%  | 3.24% | 27.18% |

In this context, some methodological strategies were performed to optimize the purification step and, consequently, to allow the quantification of hormones in this type of tissue. One of the first hypothesis of being the cause of the problem was the flow speed. From the flow essay in the SPE (section 2.2.3), it was just observed hormonal recovery in 0.3 mL.min<sup>-1</sup> and this was defined as the speed for all tests. All other flow speeds tested rendered no hormonal recovery (results not shown). The second hypothesis for this low recovery was pointed to be some carbohydrate polymerization in the presence of acetic acid. It was observed when acetic acid was used in SPE, cartridges got blocked and showed the absence of flow, even under high vacuum

pressure. This issue did not occur in the extraction solution based on formic acid. However, several methods that also use acetic acid in the extraction solution did not report the same problem [10, 20, 29, 48], indicating that this issue was related to the nature of the complex sample. When formic acid was used in the extraction and SPE solutions, it was not necessary to use the Manifold vacuum and it was defined as the main acid for extraction and purification. After overcoming the flow speed problem, the next approach was choosing the best SPE cartridge for phytohormone purification. At this point, the Oasis HLB cartridge was discarded due to its low hormonal recovery and further analysis were focused on Oasis MAX and MCX.

#### 3.2.1.1 *Oasis MAX combined with DSC-18 and variations*

When tested the use of water in place of 1 M formic acid in sample suspension before applying in Oasis SPE cartridges, interesting data were observed: ACA and ACB (composed by DSC-18 cartridge use, dryness and suspension in water, Oasis MAX with acid or basic wash, respectively) were the best proportion between hormone loss and hormone *leave on time* (when analyte eluted in elution steps), with 34.2% and 45.9% hormone an average recovery level, respectively (Figure 8). ASA and ASB (absence of use of DSC-18) presented 19.9% and 25.7% of hormone recovery level, respectively. This data supported that the process composed by C18 SPE step prior to alcoholic contend dryness, followed by suspension in water and then applied in MAX Oasis SPE (with the use of basic wash) is the best combination for phytohormone purification when it is used an Oasis MAX SPE. This reinforces the importance of Discovery DSC-18 when SPE cartridge is optimized for extracting acidic compounds with anion-exchange groups as in literature [33].

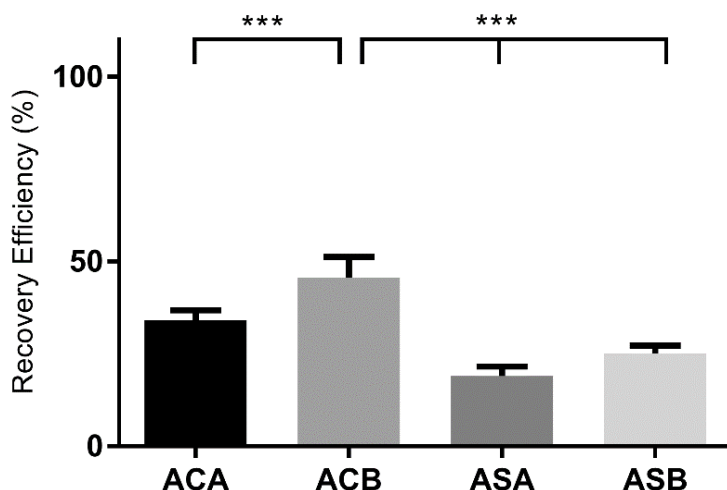


Figure 8 Comparison of phytohormone recovery efficiency among the combination of DSC-18 previously to Oasis MAX and acid or basic elution on Oasis MAX. ACA – use of DSC-18 and acid elution; ACB – use of DSC-18 and basic elution; ASA – no DSC-18 and acid elution; ASB – no DSC-18 and basic elution. \*\*\* means  $p < 0.01$

### 3.2.1.2 Oasis MCX combined with DSC-18 and variations

When using SPE Oasis MCX cartridge, it was possible to observe that CCL and CSL [which means, respectively, pass the sample into the DSC-18 cartridge, drying alcoholic content and suspension in water and applying in Oasis MCX, with long serial methanolic elution (CCL), and the same process, but without DSC-18 (CSL)] presented the highest hormone recovery level, with 89.3% and 76.8%, respectively (Figure 9). Conversely, CCN and CSN presented hormone recovery levels of 22.1% and 28.1%, respectively, composed by direct elution using ammonium hydroxide in methanol. Therefore, CSL and CCL presented better results than all other combinations, demonstrating that serial methanol elution in Elution 2 was important for raising the hormone recovery levels. It was also possible to observe that the use of SPE Discovery DSC-18 was not necessary when Oasis MCX was methanolic eluted serially in Elution 2 with increasing contents of methanol, from 0 to 100% in 20% steps (and with 0.8 M ammonium hydroxide). Aimed to simplify the Elution 2 step, a new assay evidenced that the proportion of 4:1 of methanol:water (v:v) could satisfactorily replace the serial elution (Figure 10).

The use of water instead of 1 M formic acid allowed the absence of Discovery DSC-18 use previously to SPE Oasis MCX. The extended elution step with a serial crescent concentration of ammonium hydroxide from 0.004 M to 0.4 M in methanol:water (4:1, v:v; Figure 1) proved to be the best elution mode for Oasis MCX SPE.

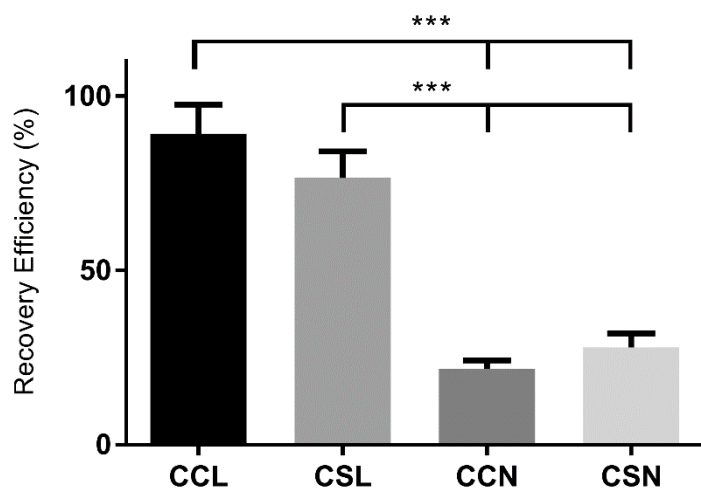


Figure 9 Comparison of hormone recovery efficiency among the combination of DSC-18 previous to Oasis MCX and direct (single step) or serial (multi step) elution on Oasis MCX. CCL- use of DSC-18 and serial elution; CSL – no DSC-18 and serial elution; CCN – use of DSC-18 and direct elution; CSN – no DSC-18 and direct elution. \*\*\*  $p < 0.01$

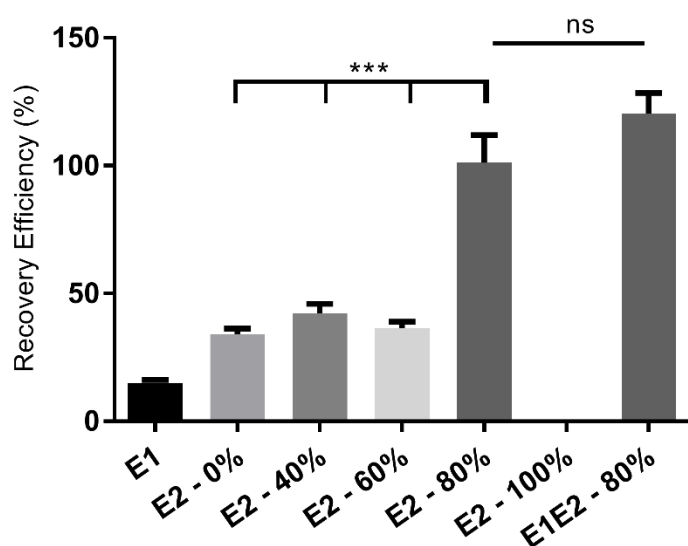


Figure 10 Comparison of hormone recovery efficiency among different methanol content in elution on Oasis MCX. E1 – elution 1; E2 0% - E2 100% - elution 2 with serial crescent methanol content; E1E2 – 80% - elution 1 combined with elution 2, directly with 80% methanol. \*\*\* means  $p < 0.01$

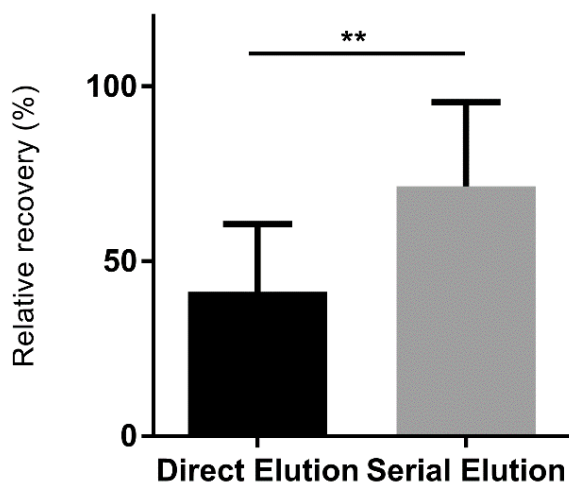


Figure 11 Comparison of hormone recovery efficiency between Direct and Serial elution of Ammonium hydroxide in Elution 2 on MCX. \*\*  $p < 0.1$

Oasis MCX and Oasis MAX cartridges were compared in order to decide the best SPE protocol to use in the purification step. Comparing the hormone recovery efficiency of MAX and MCX, it was possible to observe that the best SPE protocol was the sample dilution in water followed by its charging on Oasis MCX. In this SPE, the combination of acid wash, Elution 1 with methanol, water application, Elution 2 with methanol:water (4:1, v:v) with serial ammonium hydroxide from 0.004 M, 0.04M and 0.4 M.(Figure 12) was the very best.

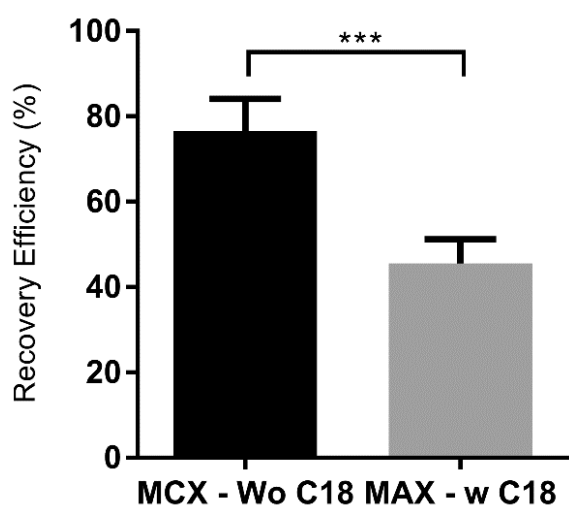


Figure 12 Comparison of hormone recovery efficiency between the best SPE protocol of MCX and MAX. \*\*\*  $p < 0.01$

### 3.2.2 PVPP treatment

During the purification process, it was possible to observe that samples were darker than expected in Elution 2 of Oasis MCX, quickly after basic elution. PVPP is a highly cross-linked modification of polyvinylpyrrolidone (PVP), insoluble in water, which is used as a refinement to extract impurities in winemaking [50,51]. Additionally, PVPP forms bonds with proteins, cleaning up samples with high concentrations of polysaccharides and polyphenolics [38,51]. In the first PVPP test (section 2.2.4), after applying 1.7% PVPP in Elute from Oasis MCX, it was possible to observe an increase in hormone recovery in relation to the non-treated sample, with 835.7% for Z, 264.8% for IAA, 230.2% for SA, 116.3% for ABA, and 595% for JA. Conversely, GA3 recovered only 96.7% (3.3% loss). For GA4, no signal was recovered in any of the treated or untreated samples (Figure 13).

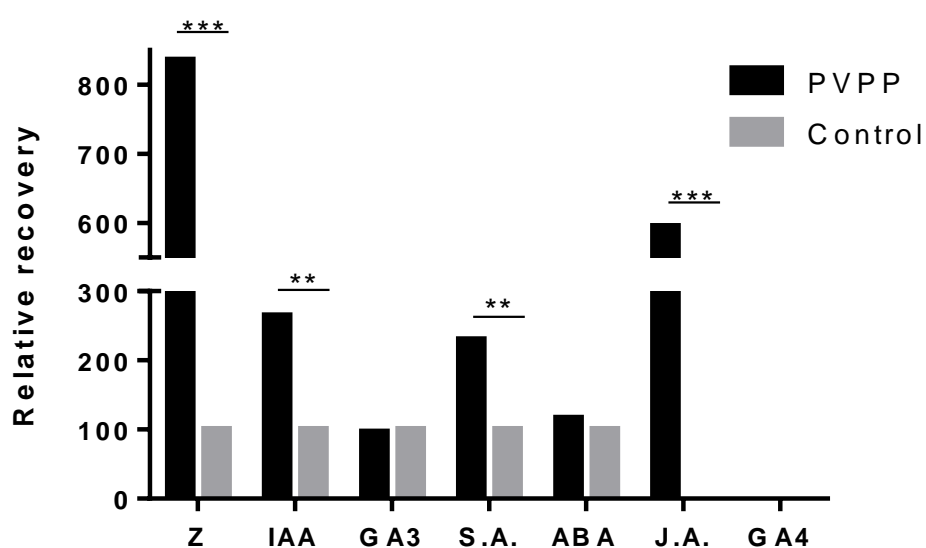


Figure 13 Hormone relative recovery of PVPP-treated sample with non-treated sample (defined as 100%). PVPP was applied after the SPE on Oasis MCX and right before the UPLC-ESI-MS/MS analysis. \*\*\* means  $p < 0.01$ ; \*\* means  $p < 0.05$ .

The second PVPP test aimed to verify whether PVPP treatment had the best efficiency in sample cleaning up. It was observed that only when treatment occurred after SPE (Figure 14), the sample was clarified. Single PVPP treatment (without SPE) was set as control, and there was no difference

between the PVPP treatment before or after the alcohol drying step (before SPE) or before and after the SPE. The PVPP treatment after SPE performed better in hormone recovery, with cleaner samples. This result was expected once pigments rose up in the SPE basic elution step. PVPP was previously used to finalize wine production and tea purification during pesticide analysis [52] in order to remove pigmentation and polyphenols, besides reports that PVPP allowed better cleaning in RNA extraction [38]. This was probably due to modifications in the structures of phenolic compounds in a basic environment, something like ninhydrin transformation, and this modification could allow pigmentation cleaning by PVPP. However, it was not expected the low hormone recovery from samples treated with PVPP before and after Oasis MCX (PVPP/SPE/PVPP). This could be due to a residue of cleaning associated to phytohormones, which could disassociate at basic pH. Looking specifically at phytohormones, ABA, GA<sub>3</sub>, and SA performed better in hormonal standard spike recovery in PVPP treatment after SPE; GA<sub>4</sub> performed better in PVPP treatment before drying and suspension; IAA and Z performed equally better in PVPP treatment before or after SPE. These results may be due to pigmentation rise in the SPE step, so the cleaning was effective only after this step. PVPP treatment before SPE cannot precipitate the contaminants probably because they were in a state that prevented them to be cleaned out. After the SPE step, with pH modulation, those molecules became susceptible to PVPP treatment, cleaning up the pigmentation. This shows how complex is a single treatment when trying to extract all hormone classes, but the majority of them performed better in recovery after SPE, which may be due to exposure to a basic environment of the ionization co-eluting molecules. This result matches with previous findings [52] and allowed us to use a one-time PVPP treatment after the SPE step. In summary, the PVPP treatment after the SPE step was the best time to perform the cleaning up of samples, also increasing phytohormone sensibility in the LC-MS phase.



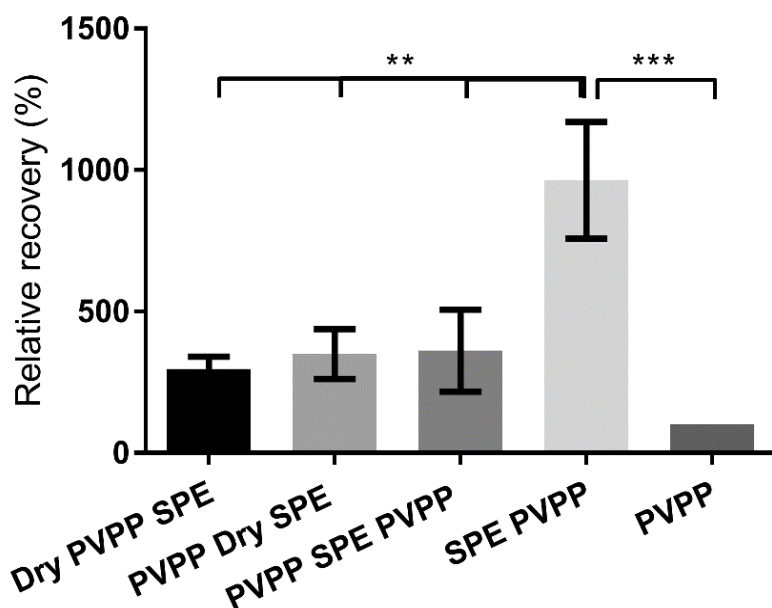


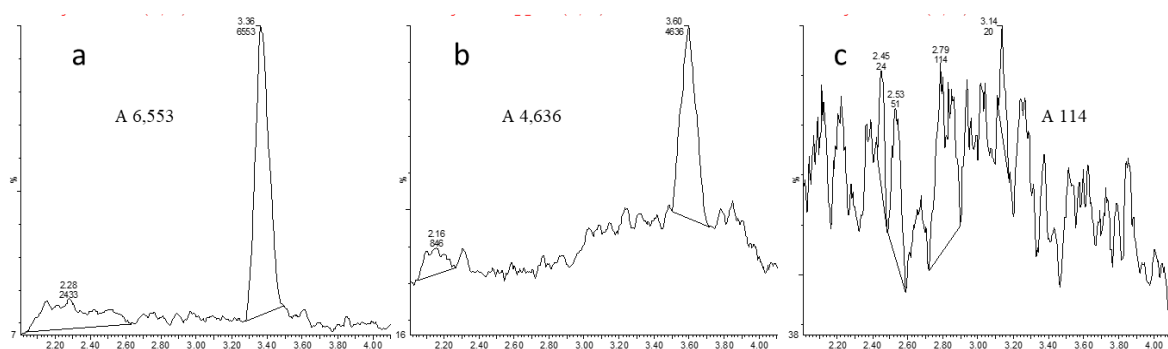
Figure 14 Relative hormone recovery comparison among diverse PVPP treatment combination with SPE MCX. 'Dry PVPP SPE' means that the PVPP treatment was applied right after the alcoholic dryness and suspension in water. 'PVPP Dry SPE' means that the PVPP treatment was applied right before the alcoholic dryness and suspension in water. \*\*\* means  $p < 0.001$  and \*\* means  $p < 0.01$

After the adjustments in the purification step, the final protocol was defined as follows: supernatants from the extraction are completely dried in a vacuum centrifuge (6,700 x g at 40 °C under vacuum) and suspended in 1.5 ml of water. Oasis MCX cartridge is wetted with 5 ml of methanol and conditioned with 5 ml of water. Sample is loaded on SPE and washed with 3 ml of 1 M formic acid. The elution 1 is composed of 4 ml of methanol and, then, 1 ml of water. Elution 2 is composed by 1.5 ml of 0.004 M, 1.5 ml of 0.04 M, and 2 ml of 0.4 M ammonium hydroxide in methanol:water (4:1, v:v). Elutions of 10 ml are combined and treated with 1.7 % PVPP mass (nearly 0.17 g), vortexed vigorously for 30 s and kept at -20 °C for 30 min. After that, vortexing and cold incubation are repeated once and then the samples are centrifuged (10,000 x g for 45 min at 4 °C) to precipitate PVPP and supernatants are collected for the analysis by UPLC-ESI-MS/MS.

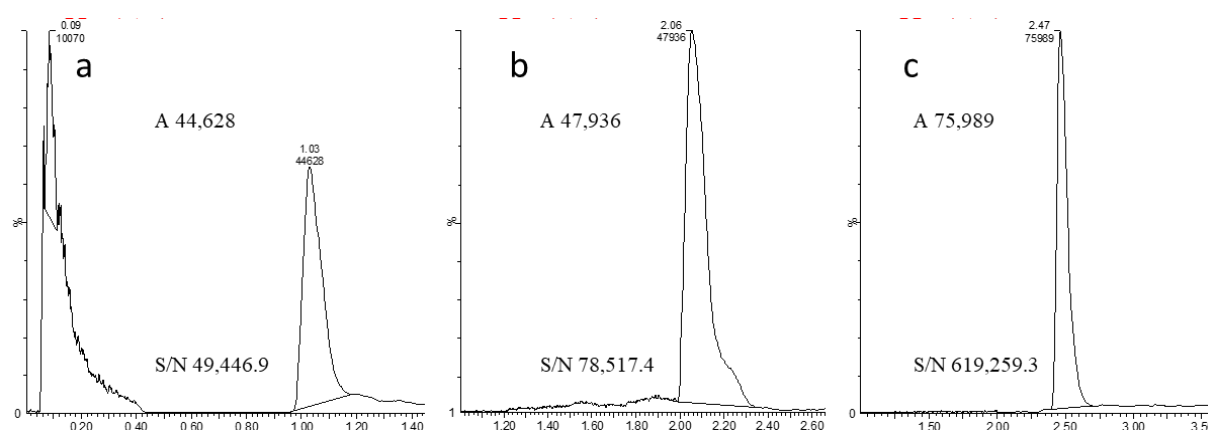
### 3.3 *Chromatography and detection steps*

The chromatography and detection steps were also improved in order to reduce the negative effects of the matrix and to optimize the sensibility of detecting hormones in these complex plant extracts. UPLC-ESI-MS/MS is currently the standard quantification method used worldwide [29,53,54]. Despite this capability, many works emphasize that this system may present some ionization and detection problems caused by matrix compounds that co-elute with target analytes, especially when using the ESI in positive mode [14]. Therefore, some chromatographic and ionization modification tests were performed to guarantee more sensitivity in the quantification.

In order to identify the best eluents and additives for the mobile phase, some assays were performed (Section 2.3.2). The best A and B solutions were 0.1% formic acid in water (solution A) and 0.1% formic acid in acetonitrile (solution B). However, when 0.1% formic acid was applied in water (A) and acetonitrile/methanol (1:1 blend, B), the performance was inferior than the first condition. Furthermore, almost all target analyte standards were lost when A (water) and B (acetonitrile) solutions were added with ammonium hydroxide. The elution of IAA using these three different A and B solution combinations is shown in LC according to Figure 85. Similar results were previously observed when using a binary solvent system composed of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) [5,23,55]. Sometimes the formic acid at 0.1% was substituted by acetic acid at 0.05% [45,56], but formic acid showed better graph resolutions in our tests (data not shown). The choice to perform LC under an acidic pH and varying the organic profile seemed to be the most used method since analytes were retained in their neutral form in the column for a longer time. Additionally, they were eluted at a higher organic concentration in the LC gradient, and after the matrix elution that cause ME, making the analysis more robust.



**Figure 85** Comparison of IAA quantification among different A and B solution combinations on LC. G2 was used as the main gradient. a – 0.1% formic acid in water (A) and acetonitrile (B); b – 0.1% formic acid in water (A) and acetonitrile:methanol (1:1, v:v) (B); c – 0.05% ammonium hydroxide in water (A) and acetonitrile (B). The X axis represents the time of LC column retention and the Y axis represents the IAA detection in the MS/MS system. A represents the calculated area of IAA detection in the right column retention time.

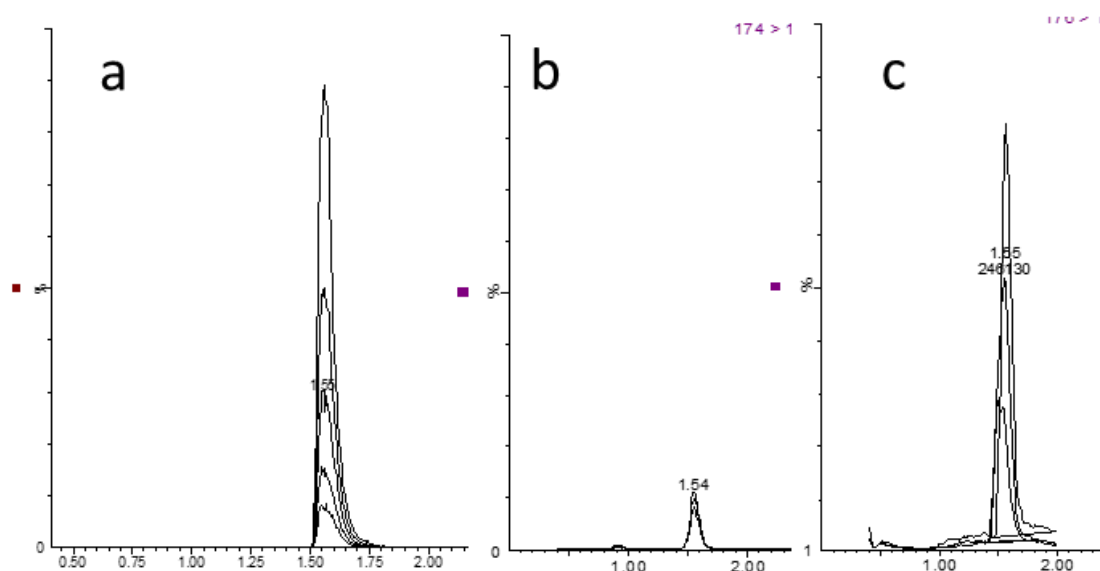


**Figure 96** Comparison among Z quantification in diverse gradients. A - gradient G1; B - gradient G2; C - gradient G3. The X axis represents the time of LC column retention and the Y axis represents the IAA detection in the MS/MS system. A represents the calculated area of Z detection in the right column retention time.

In order to increase the capacity of chromatographic separation, three gradients were tested in LC. The first condition (G1) was based on preliminary tests and only with a standard solution (no ME). In G2, the ramp was increased to 4.2 min in 100% B solution (acetonitrile with 0.1% formic acid). However, these methods were lower in the separation and restriction capacity of the ME when compared to the method that employed the longest time for the increment

of B solution (G3), which reached 100% at 6.5 min. This last LC condition performed the best results considering peak intensity, area and signal/noise ratio (Figure 96). This data confirmed that less inclined gradients allow a better component separation, decreasing ME and increasing sensitivity to phytohormone detection, since this approach allows a better separation of analyte from coelute ionization contaminants despite consuming more time and resources [18].

These two modifications allowed the recovery of UPLC-ESI-MS/MS sensitivity to hormones in samples experiencing ME. The ME overcomes the signal of the hormonal standard curve (visible in Figure 107 b). After the new chromatographic parameter adjustments and the employment of previous steps turned the method much more advantageous concerning the ME (Fig 17 c).



*Figure 107 Comparison of 3 stages of IAA standard curve affected by ME in quantification. A - curve in blank solution and initial UPLC-MS/MS parameters (without ME); B - curve in sample and initial UPLC-MS/MS parameters (with ME); C - curve in sample and new UPLC-MS/MS parameters (ME overcome). The X axis represents the time of LC column retention and the Y axis represents the IAA detection in the MS/MS system.*

The final detection/quantification step was composed by a concentration of samples to less than 200  $\mu$ l, filtering through a 0.2  $\mu$ m filter and analysis in a 10 min reading time (G3). The final adjustments were used to establish standard curves. Table 5 shows the details of each phytohormone used. The

results of Limit of Quantification (LOQ, when the signal/noise is greater than 10) were relatively higher than other methods for phytohormone detection and quantification, especially for GA<sub>4</sub>, IAA and SA [3, 33, 57]. Those methods were performed with a clean reading solution (methanol, acetonitrile or a mix of them), and our method was set by reading samples, full of ME. Those methods could not allow the quantification of phytohormones in our conditions. So, considering this, our method was sensitive enough to quantify the hormones even under high ME and in little quantities.

Table 5: Phytohormone standard response characteristic in UPLC with the last adjustments developed in LC and MS. The curves were defined with apple tree dormant buds as matrix.

| Phytohormone standard | Mean retention time | Peak area (25 ng.ml <sup>-1</sup> ) | Equation of calibration curve | R <sup>2</sup> | Linear range (ng.ml <sup>-1</sup> ) | LOQ (ng) |
|-----------------------|---------------------|-------------------------------------|-------------------------------|----------------|-------------------------------------|----------|
| ABA                   | 3.6                 | 842.450                             | y= 26.4004x+182.44            | 0.770          | 5–1500                              | 6.910    |
| GA <sub>3</sub>       | 2.6                 | 764.462                             | y= 29.8114x+19.1774           | 0.975          | 5–1500                              | 0.643    |
| GA <sub>4</sub>       | 5.5                 | 356.126                             | y= 11.8487x+59.9083           | 0.927          | 5–1500                              | 5.056    |
| IAA                   | 3.1                 | 3,766.908                           | y= 115.374x+882.558           | 0.993          | 5–1500                              | 7.650    |
| JA                    | 4.3                 | 947.402                             | y= 31.0027x+172.335           | 0.999          | 5–1500                              | 5.560    |
| SA                    | 3.2                 | 3,677.688                           | y= 95.8139x+1282.34           | 0.970          | 5–1500                              | 13.384   |
| Z                     | 1.9                 | 18,200.71                           | y= 640.84x+2179.71            | 0.989          | 5–1500                              | 3.401    |

### 3.3 ME with Adjusted Protocol

ME is the greatest issue in qualitative and quantitative analysis of phytohormones or other low-amount metabolites, corrupting results by masking or intensifying differences of similar but not identical samples [15,17]. This issue is the main limiting factor in the wide use of UPLC-MS/MS of new complex samples, since the usual samples are well defined and this issue was already overcome [24,58].

The ME measurement in the *Recovery Efficiency test* demonstrated that there was a strong impact from ME in apple dormant buds: GA<sub>4</sub>, ABA, IAA and SA exhibited 84%, 82%, 75% and 125% recovery, respectively, forming the less impacted hormonal group. GA<sub>3</sub> showed a 9% recovery, JA recovered 0.1%, and

Z exhibited a 3,193.9% recovery, and this second group is the strongly impacted group by ME. These highly disturbed results were becoming less interfered by ME with the progress of method adaptation to apple dormant bud samples. The advances in extraction, purification and quantification allowed a better separation of phytohormones from the sample interference, especially in purification step and gradient in LC. Another important point was the build calibration curves of standards to LC-MS in a final sample mixture and the LC reading solution. This approach was shown to be very useful to minimize ME [17,58]. After all development and modifications in the method, the Recovery Efficiencies for the phytohormones were 89.5 % to GA3, 101.4 % to GA4, 109.5 % to Z, 112.7 % for SA, 90.9 % to IAA, 103.9 % to JA and 104.2 % to ABA. Recoveries over 100% are due to standard spiked recovery plus the endogenous hormone recovery. This happens when the endogenous is under LOQ, so is not possible to subtract the endogenous from the final recovery calculation.

Aiming to prove the applicability of the method to other tissues, the protocol here in described (Table 6) was tested on tissues from different plants and in distinct metabolic developmental stages. Most of these samples were lignified, dormant or highly pigmented tissues. In these tests we also included some tissues that were not highly lignified or pigmented like *Araucaria angustifolia* sprouts or *Podocarpus lambertii* somatic embryos, rich in waxes. In Table 6 is represented the values obtained with this method on those samples (described in section 2.4.1) and compared with works that had already quantified these hormones in those samples. When both methods allowed the quantification of hormones, the box was colored: Blue - when our method quantified more hormone, and Gray - when it quantified less.

Table 6 Comparison of the method developed in this present work for hormonal quantification with other, published methods [59, 60, 61]. Hormones analyzed by both methods are colored in blue when our method quantified more hormones, gray when quantified less. The value from our test is in upper part of the box and the others value is with an asterisk.

| ng/g FW                   | ABA                          | GA <sub>3</sub>     | GA <sub>4</sub>            | IAA                      | JA                 | SA                  | Z                    |
|---------------------------|------------------------------|---------------------|----------------------------|--------------------------|--------------------|---------------------|----------------------|
| <b>Apple bud</b>          | 38.48±3.41                   | 9.63±6.92           | 295.68±4.74                | 0.00                     | 2634.13<br>±583.18 | 2622.26<br>±273.49  | 0.00                 |
| <b>Grape bud</b>          | 354.04±5.82                  | 2244.06<br>±42.09   | 509.73±12.25               | 1628.49±29.04            | 0.00               | 63602.70<br>±783.51 | 26.29<br>±1.68       |
| <b>Pear bud</b>           | 234.52±13.46<br>0.087±0.005* | 1.71±0.42           | 20.61±3.89<br>0.037±0.002* | 2.70±1.20<br>0.05±0.001* | 253.81<br>±39.68   | 106.24<br>±8.01     | 2.58±0.09<br>0.01±0* |
| <b>Rose flower bud</b>    | 396.76±22.85<br>450.00±8.00* | 0.00<br>40.00±4.00* | 170.53±6.83<br>0.85±0.10*  | 0.00<br>24.50±4.90*      | 2162.21<br>±279.51 | 702.93<br>±12.03    | 0.05±0.01            |
| <b>Pine embryo</b>        | 593.78±170.20<br>0.0005*     | 0.00                | 0.00                       | 0.00<br>0.00*            | 0.00               | 920.14<br>±13.09    | 61.92<br>±4.72       |
| <b>Pine seed 1</b>        | 15674.13<br>±472.18          | 133.00<br>±21.09    | 542.95±4.82                | 382.95±14.62             | 0.00               | 10961.03<br>±371.27 | 13.00<br>±1.01       |
| <b>Pine seed 4</b>        | 551.00±2.85                  | 555.93±8.42         | 644.48±22.81               | 0.00                     | 0.00               | 10537.12<br>±98.37  | 0.00                 |
| <b>Banana green fruit</b> | 426.83±21.14                 | 18.96±3.63          | 363.22±1.64                | 21.15±0.94               | 101.79<br>±9.03    | 1651.11<br>±62.5    | 8.30±0.09            |

The analysis of pear floral buds resulted in quantification of hormones similar to other work, with minor differences [59], with 3 more hormones quantified in this method. Rose floral bud performed similar too, despite our sample be a recently opened bud and the other work be a fully opened flower [60] and again our method allowed the quantification of more hormones. The pine somatic embryos performed better in our work, with more hormones and greater quantification with less sample mass then the other [61]. Other samples did not have a register for hormone quantification, especially dormant buds or immature fruits, and in this work they were quantified successfully. In this

method it was possible to quantify more hormones with better results in common samples and novelty in not usual tissues.

The development of a fast, easy, sensitive, selective, cheap and high efficient quantification method for phytohormones in a real complex matrix is almost impossible, but some modifications and adaptations could overcome many of the problems and allowed us to establish such method. The present method (summarized in Figure 18) enables the quantification of seven phytohormones even in low quantities in lignified samples [26], with reproducibility. This method is efficient enough to process 48 samples, from field harvest to final quantification, in approximately 50 hours. The total reading quantification time in UPLC/ESI-MS/MS with MRM allowed each sample to be analyzed in 10 min.

#### **4 Conclusion**

In this work, a new protocol for hormonal quantification in lignified and complex structures of wood perennial species by UPLC-ESI-MS/MS is described (Figure 18). Using the parameters in the extraction, purification and chromatographic steps the ME was reduced, enabling the monitoring of seven hormones (GA<sub>3</sub>, GA<sub>4</sub>, ABA, SA, JA, Z, and IAA). In addition, the same method allowed the quantification of these hormones in six different types of tissues, which shows a broad applicability (Table 5). However, due to the complexity of the plant tissues and the contrasts that exist in the analytical capacity of the equipment, additional but minor adjustments may be necessary for each case. This approach will be undoubtedly useful to quantify phytohormones in complex matrices and new types of samples, allowing the advance of physiology and molecular knowledge of plant metabolism, especially in dormant structures and organs.



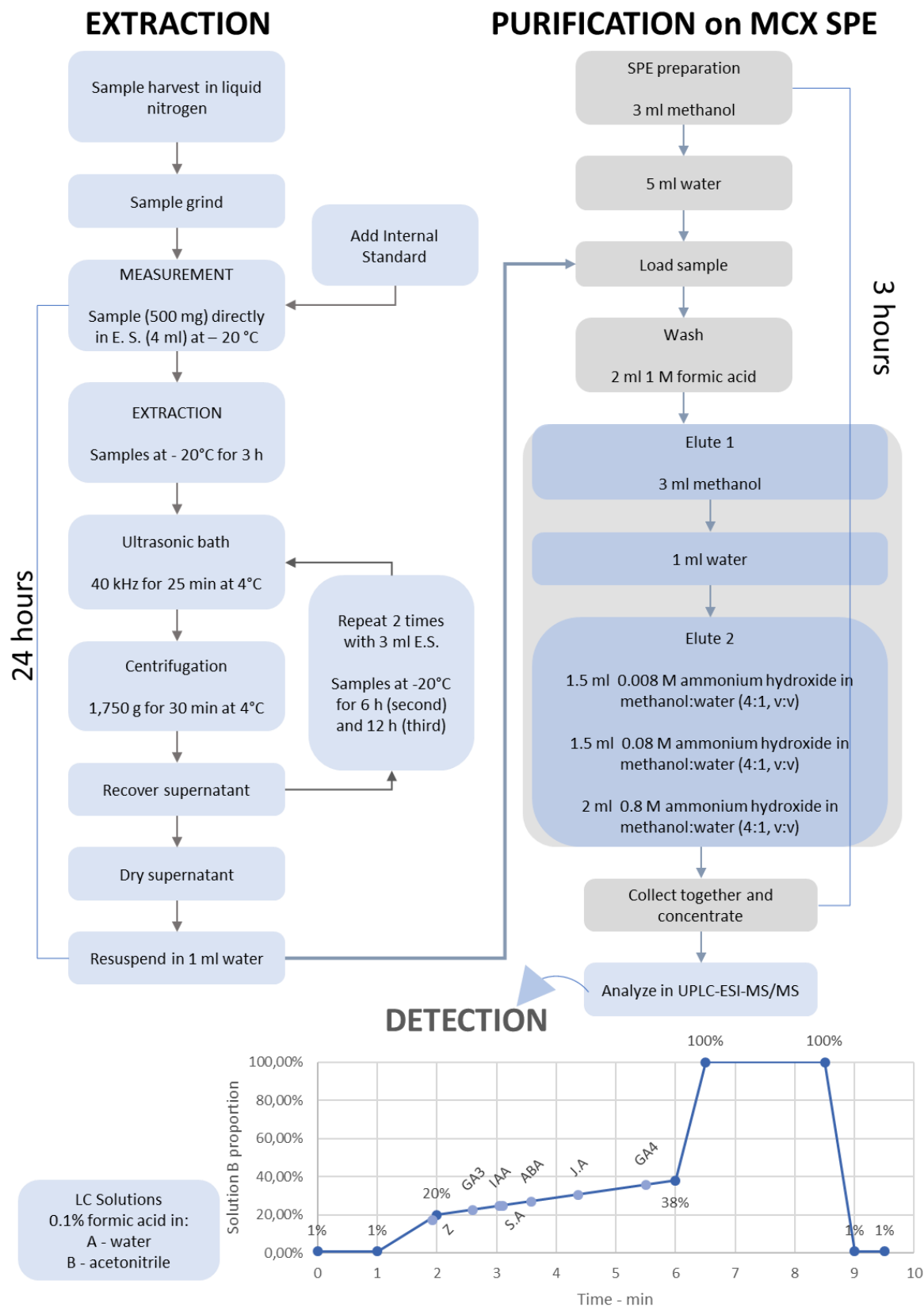


Figure 18 Summary of the protocol developed in this work for the extraction and quantification of seven phytohormones in different types of lignified and non-lignified tissues.

**Conflicts of interest:** The authors declare no conflict of interest.

**Acknowledgements / Funding:** This work was supported by the Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Financiadora de Estudos e Projetos (FINEP).

## References

- [1] P.J. Davies, *The Plant Hormones: Their Nature, Occurrence, and Functions*, in: *Plant Horm.*, Springer Netherlands, Dordrecht, 2010: pp. 1–15. doi:10.1007/978-1-4020-2686-7\_1.
- [2] J.J. Ross, D.E. Weston, S.E. Davidson, J.B. Reid, Plant hormone interactions: How complex are they?, *Physiol. Plant.* 141 (2011) 299–309. doi:10.1111/j.1399-3054.2011.01444.x.
- [3] M. Müller, S. Munné-Bosch, Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry., *Plant Methods.* 7 (2011) 37. doi:10.1186/1746-4811-7-37.
- [4] E.A. Dun, P.B. Brewer, C.A. Beveridge, Strigolactones : discovery of the elusive shoot branching hormone, (2009). doi:10.1016/j.tplants.2009.04.003.
- [5] X. Pan, R. Welti, X. Wang, Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry, *Phytochemistry.* 69 (2008) 1773–1781. doi:10.1016/j.phytochem.2008.02.008.
- [6] P.J. Davies, *Plant Hormones*, Springer Netherlands, Dordrecht, 2010. doi:10.1007/978-1-4020-2686-7.
- [7] S. Fahad, S. Hussain, A. Matloob, F.A. Khan, A. Khaliq, S. Saud, S. Hassan, D. Shan, F. Khan, N. Ullah, M. Faiq, M.R. Khan, A.K. Tareen, A. Khan, A. Ullah, N. Ullah, J. Huang, Phytohormones and plant responses to salinity stress: a review, *Plant Growth Regul.* 75 (2015) 391–404. doi:10.1007/s10725-014-0013-y.
- [8] B. Lacombe, P. Achard, Long-distance transport of phytohormones through the plant vascular system, *Curr. Opin. Plant Biol.* 34 (2016) 1–8. doi:10.1016/j.pbi.2016.06.007.
- [9] D.E. Weston, J.B. Reid, J.J. Ross, Auxin regulation of gibberellin biosynthesis in the roots of pea ( *Pisum sativum* ), *Funct. Plant Biol.* 36 (2009) 362–369. doi:10.1071/FP08301.
- [10] M. Tanaka, K. Takei, M. Kojima, H. Sakakibara, H. Mori, Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance, *Plant J.* 45 (2006) 1028–1036. doi:10.1111/j.1365-313X.2006.02656.x.
- [11] S. Gazzarrini, P. McCourt, Genetic interactions between ABA, ethylene and sugar signaling pathways, *Curr. Opin. Plant Biol.* 4 (2001) 387–391. doi:10.1016/S1369-5266(00)00190-4.
- [12] S. Wilkinson, W.J. Davies, Drought, ozone, ABA and ethylene: new insights from cell to plant to community, *Plant, Cell Environ.* 33 (2010) 510–525. doi:10.1111/j.1365-3040.2009.02052.x.
- [13] B.K. Matuszewski, M.L. Constanzer, Matrix Effect in Quantitative LC / MS / MS Analyses of Biological Fluids : A Method for Determination of Finasteride in Human Plasma at Picogram Per Milliliter Concentrations, 2700 (1998) 1001–1008. doi:10.1021/ac971078+.
- [14] R. Dams, M.A. Huestis, W.E. Lambert, C.M. Murphy, Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: Influence of ionization type, sample preparation, and biofluid, *J. Am. Soc. Mass Spectrom.* 14 (2003) 1290–1294. doi:10.1016/S1044-0305(03)00574-9.

- [15] P. Vats, S.M. Verma, Mechanistic Evaluation of Matrix Effect on Three Different Model of Mass Spectrometer by Using a Model Drug, *J. Anal. Bioanal. Tech.* 7 (2016) 1–11. doi:10.4172/2155-9872.1000314.
- [16] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, *Anal. Chem.* 75 (2003) 3019–3030. doi:10.1021/ac020361s.
- [17] H. Truffelli, P. Palma, G. Famiglini, A. Cappiello, An overview of matrix effects in liquid chromatography-mass spectrometry, *Mass Spectrom. Rev.* 30 (2011) 491–509. doi:10.1002/mas.20298.
- [18] E. Chambers, D.M. Wagrowski-diehl, Z. Lu, J.R. Mazzeo, Systematic and comprehensive strategy for reducing matrix effects in LC / MS / MS analyses, 852 (2007) 22–34. doi:10.1016/j.jchromb.2006.12.030.
- [19] A. D. McNaught and A. Wilkinson, Matrix Effect, in: *IUPAC Compend. Chem. Terminol.*, IUPAC, Research Triangle Park, NC, 2006: p. 3759. doi:10.1351/goldbook.M03759.
- [20] H. Guo, Z.S. Breitbach, D.W. Armstrong, Reduced matrix effects for anionic compounds with paired ion electrospray ionization mass spectrometry, *Anal. Chim. Acta.* 912 (2016) 74–84. doi:10.1016/j.aca.2016.01.038.
- [21] S. Wang, M. Cyronak, E. Yang, Does a stable isotopically labeled internal standard always correct analyte response?, *J. Pharm. Biomed. Anal.* 43 (2007) 701–707. doi:10.1016/j.jpba.2006.08.010.
- [22] D. Remane, M.R. Meyer, D.K. Wissenbach, H.H. Maurer, Ion suppression and enhancement effects of co-eluting analytes in multi-analyte approaches: systematic investigation using ultra-high-performance liquid chromatography/mass spectrometry with atmospheric-pressure chemical ionization or electrospray ionization, *Rapid Commun. Mass Spectrom.* 24 (2010) 3103–3108. doi:10.1002/rcm.4736.
- [23] D.M. Kasote, R. Ghosh, J.Y. Chung, J. Kim, I. Bae, H. Bae, Multiple Reaction Monitoring Mode Based Liquid Chromatography-Mass Spectrometry Method for Simultaneous Quantification of Brassinolide and Other Plant Hormones Involved in Abiotic Stresses, *Int. J. Anal. Chem.* 2016 (2016) 1–8. doi:10.1155/2016/7214087.
- [24] C. Bylda, R. Thiele, U. Kobold, D.A. Volmer, Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS, *Analyst.* 139 (2014) 2265. doi:10.1039/c4an00094c.
- [25] K. Ljung, G. Sandberg, T. Moritz, *Methods of Plant Hormone Analysis*, in: *Plant Horm.*, Springer Netherlands, Dordrecht, 2010: pp. 717–740. doi:10.1007/978-1-4020-2686-7\_31.
- [26] V. Szecskó, K. Hrotkó, E. Stefanovits-Bányai, Phenolic compounds, bud dormancy, and rooting ability of plum hardwood cuttings, *Acta Hort.* (2004) 679–687. doi:10.17660/ActaHortic.2004.658.104.
- [27] EPPO (1984), EPPO crop growth stage keys—apple and pear., 1984. doi:10.1111/j.1365-2338.1999.tb01431.x.
- [28] V. da S. Falavigna, D.D. Porto, V. Buffon, M. Margis-Pinheiro, G. Pasquali, L.F. Revers, Differential Transcriptional Profiles of Dormancy-Related Genes in Apple Buds, *Plant Mol. Biol. Report.* 32 (2014) 796–813. doi:10.1007/s11105-013-0690-0.
- [29] A. Malachová, M. Sulyok, E. Beltrán, F. Berthiller, R. Krska, Optimization and validation of a quantitative liquid chromatography – tandem mass spectrometric method covering 295 bacterial and fungal metabolites including all regulated mycotoxins in four model food matrices, *J. Chromatogr. A.* 1362 (2014) 145–156. doi:10.1016/j.chroma.2014.08.037.
- [30] M.A. Seif El-Yazal, S.A. Seif El-Yazal, M.M. Rady, Exogenous dormancy-breaking

- substances positively change endogenous phytohormones and amino acids during dormancy release in “Anna” apple trees, *Plant Growth Regul.* 72 (2014) 211–220. doi:10.1007/s10725-013-9852-1.
- [31] G.J. de Klerk, H. Guan, P. Huisman, S. Marinova, Effects of phenolic compounds on adventitious root formation and oxidative decarboxylation of applied indoleacetic acid in *Malus* “Jork 9,” *Plant Growth Regul.* 63 (2011) 175–185. doi:10.1007/s10725-010-9555-9.
- [32] P. Ivanov Dobrev, M. Kamínek, Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction, *J. Chromatogr. A.* 950 (2002) 21–29. doi:10.1016/S0021-9673(02)00024-9.
- [33] P.I. Dobrev, R. Vankova, Quantification of Abscisic Acid, Cytokinin, and Auxin Content in Salt-Stressed Plant Tissues, in: S. Shabala, T.A. Cuin (Eds.), *Plant Salt Toler. Methods Protoc.*, Humana Press, Totowa, NJ, 2012: pp. 251–261. doi:10.1007/978-1-61779-986-0\_17.
- [34] P.I. Dobrev, L. Havlicek, M. Vágner, J. Malbeck, M. Kamínek, Purification and determination of plant hormones auxin and abscisic acid using solid phase extraction and two-dimensional high performance liquid chromatography, *J. Chromatogr. A.* 1075 (2005) 159–166. doi:10.1016/j.chroma.2005.02.091.
- [35] R. Zhou, T.M. Squires, S.J. Ambrose, S.R. Abrams, A.R.S. Ross, A.J. Cutler, Rapid extraction of abscisic acid and its metabolites for liquid chromatography-tandem mass spectrometry, *J. Chromatogr. A.* 1010 (2003) 75–85. doi:10.1016/S0021-9673(03)01029-X.
- [36] Supelco; Sigma-Aldrich Co., *Bulletin 910 - Guide to Solid Phase Extraction*, (1998).
- [37] S. Technologies, A. Agilent Corp., *SPE Method Development Tips and Tricks*, (2009).
- [38] Y. Zeng, T. Yang, RNA isolation from highly viscous samples rich in polyphenols and polysaccharides, *Plant Mol. Biol. Report.* 20 (2002) 417–417. doi:10.1007/BF02772130.
- [39] S.K.S. Amoah, L.P. Sandjo, J.M. Kratz, M.W. Biavatti, Rosmarinic Acid - Pharmaceutical and Clinical Aspects, *Planta Med.* 82 (2016) 388–406. doi:10.1055/s-0035-1568274.
- [40] C. Castelluccio, G. Paganga, N. Melikian, G. Paul Bolwell, J. Pridham, J. Sampson, C. Rice-Evans, Antioxidant potential of intermediates in phenylpropanoid metabolism in higher plants, *FEBS Lett.* 368 (1995) 188–192. doi:10.1016/0014-5793(95)00639-Q.
- [41] K. Schwarz, W. Ternes, Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*, *Z. Lebensm. Unters. Forsch.* 195 (1992) 99–103. doi:10.1007/BF01201766.
- [42] W.W. Cleland, Dithiothreitol, a New Protective Reagent for SH Groups, *Biochemistry.* 3 (1964) 480–482. doi:10.1021/bi00892a002.
- [43] J.C. Lukesh, M.J. Palte, R.T. Raines, A Potent, Versatile Disulfide-Reducing Agent from Aspartic Acid, *J. Am. Chem. Soc.* 134 (2012) 4057–4059. doi:10.1021/ja211931f.
- [44] E.B. Getz, M. Xiao, T. Chakrabarty, R. Cooke, P.R. Selvin, A Comparison between the Sulfhydryl Reductants Tris(2-carboxyethyl)phosphine and Dithiothreitol for Use in Protein Biochemistry, *Anal. Biochem.* 273 (1999) 73–80. doi:10.1006/abio.1999.4203.
- [45] S.C. Farrow, R.N. Emery, Concurrent profiling of indole-3-acetic acid, abscisic acid, and cytokinins and structurally related purines by high-performance-liquid-chromatography tandem electrospray mass spectrometry, *Plant Methods.* 8 (2012) 42. doi:10.1186/1746-4811-8-42.
- [46] F. Matsuda, H. Miyazawa, K. Wakasa, H. Miyagawa, Quantification of indole-3-acetic acid and amino acid conjugates in rice by liquid chromatography-electrospray ionization-tandem mass spectrometry, *Biosci. Biotechnol. Biochem.* 69 (2005) 778–783. doi:10.1271/bbb.69.778.
- [47] M. Gilar, A. Jaworski, T.S. McDonald, Solvent selectivity and strength in reversed-phase

- liquid chromatography separation of peptides, *J. Chromatogr. A.* 1337 (2014) 140–146. doi:10.1016/j.chroma.2014.02.048.
- [48] K. Cui, Y. Lin, X. Zhou, S. Li, H. Liu, F. Zeng, F. Zhu, G. Ouyang, Z. Zeng, Comparison of sample pretreatment methods for the determination of multiple phytohormones in plant samples by liquid chromatography-electrospray ionization-tandem mass spectrometry, *Microchem. J.* 121 (2015) 25–31. doi:10.1016/j.microc.2015.02.004.
- [49] C. Delatorre, A. Rodríguez, L. Rodríguez, J.P. Majada, R.J. Ordás, I. Feito, Hormonal profiling: Development of a simple method to extract and quantify phytohormones in complex matrices by UHPLC–MS/MS, *J. Chromatogr. B.* 1040 (2017) 239–249. doi:10.1016/j.jchromb.2016.11.007.
- [50] I.M. Pereira, R.H. Moretti, Caracterização física, química e sensorial do vinho branco seco sauvignon blanc tratado com polivinilpirrolidona (PVPP), *Ciência E Tecnol. Aliment.* 17 (1997) 192–195. doi:10.1590/S0101-20611997000200024.
- [51] I.O.V.W. International Organisation of Vine and Wine, International Code of Oenological Practices, PARIS, 2017. <http://www.oiv.int/public/medias/5119/code-2017-en.pdf>.
- [52] R. Hou, W. Jiao, Y. Xiao, J. Guo, Y. LV, H. Tan, J. Hu, X. Wan, Novel use of PVPP in a modified QuEChERS extraction method for UPLC-MS/MS analysis of neonicotinoid insecticides in tea matrices, *Anal. Methods.* 7 (2015) 5521–5529. doi:10.1039/C5AY00957J.
- [53] J.M. Armenta, D.F. Cortes, J.M. Pisciotta, J.L. Shuman, K. Blakeslee, D. Rasoloson, O. Ogunbiyi, D.J. Sullivan, V. Shulaev, Sensitive and rapid method for amino acid quantitation in malaria biological samples using AccQ.Tag ultra performance liquid chromatography-electrospray ionization-MS/MS with multiple reaction monitoring., *Anal. Chem.* 82 (2010) 548–558. doi:10.1021/ac901790q.
- [54] Y. Xiang, X. Song, J. Qiao, Y. Zang, Y. Li, Y. Liu, C. Liu, An ultrahigh-performance liquid chromatography method with electrospray ionization tandem mass spectrometry for simultaneous quantification of five phytohormones in medicinal plant *Glycyrrhiza uralensis* under abscisic acid stress, *J. Nat. Med.* 69 (2015) 278–286. doi:10.1007/s11418-015-0889-5.
- [55] M. Schäfer, M. Reichelt, I. Baldwin, S. Meldau, Cytokinin Analysis: Sample Preparation and Quantification, *Bio-Protocol.* 4 (2014) 1–8. doi:10.21769/BioProtoc.1167.
- [56] F. Matsuda, H. Miyazawa, K. Wakasa, H. Miyagawa, Quantification of Indole-3-Acetic Acid and Amino Acid Conjugates in Rice by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry, *Biosci. Biotechnol. Biochem.* 69 (2005) 778–783. doi:10.1271/bbb.69.778.
- [57] F. Du, G. Ruan, H. Liu, Analytical methods for tracing plant hormones, *Anal. Bioanal. Chem.* 403 (2012) 55–74. doi:10.1007/s00216-011-5623-x.
- [58] F. Gosetti, E. Mazzucco, D. Zampieri, M.C. Gennaro, Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry, *J. Chromatogr. A.* 1217 (2010) 3929–3937. doi:10.1016/j.chroma.2009.11.060.
- [59] Q. Niu, Y. Zong, M. Qian, F. Yang, Y. Teng, Simultaneous quantitative determination of major plant hormones in pear flowers and fruit by UPLC/ESI-MS/MS, *Anal. Methods.* 6 (2014) 1766. doi:10.1039/c3ay41885e.
- [60] X. Guo, C. Yu, L. Luo, H. Wan, N. Zhen, T. Xu, J. Tan, H. Pan, Q. Zhang, Transcriptome of the floral transition in *Rosa chinensis* “Old Blush,” *BMC Genomics.* 18 (2017) 199. doi:10.1186/s12864-017-3584-y.
- [61] N. Steiner, C. Santa-Catarina, V. Silveira, E.I.S. Floh, M.P. Guerra, Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures, *Plant Cell. Tissue Organ Cult.* 89 (2007) 55–62. doi:10.1007/s11240-007-9216-5.

## 4 CAPÍTULO II

### **Hormonal Quantification in Dormant Buds of Apple Trees under the Influence of Different Rootstocks**

*Manuscrito a ser submetido ao periódico 'New Phytologist' (Fl: 7,21)*

## **Title**

Hormonal Quantification in Dormant Buds of Apple Trees under the Influence of Different Rootstocks.

## **Authors**

Julio de Andrade Garighan<sup>1,2</sup>, Daniel Antunes Souza<sup>2</sup>, Giancarlo Pasquali<sup>1</sup>, Luís Fernando Revers<sup>2, §</sup>, Henrique Pessoa dos Santos<sup>2, §</sup>

<sup>1</sup>Graduate Program in Cell and Molecular Biology, Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS 91501-970, Brazil

<sup>2</sup> Embrapa Uva e Vinho, Bento Gonçalves, RS, 95701-008, Brazil

§Corresponding authors

Dr. Henrique Pessoa dos Santos

Laboratory of Plant Physiology, Embrapa Uva e Vinho, Rua Livramento, 515, P.O. Box 130, 95701-008, Bento Gonçalves, RS, Brazil

**Tel:** +55 54 3455 8126

**Fax:** +55 54 3455 8127

**E-mail:** henrique.p.santos@embrapa.br

Dr. Luís Fernando Revers

Laboratory of Plant Molecular Genetics, Embrapa Uva e Vinho, Rua Livramento, 515, P.O. Box 130, CEP: 95701-008, Bento Gonçalves, RS, Brazil

**Tel:** +55 54 3455 8039

**Fax:** +55 54 3451 2792

**E-mail:** luis.revers@embrapa.br

## Summary

1. Apple trees (*Malus x domestica* Borkh.) enter endodormancy in autumn and require chilling (<7.2°C) accumulation to overcome it. Gene expression studies of dormant buds indicate that hormones are crucial factors in endo-ecodormancy transition and in budbreak. The endodormancy is regulated by the bud itself, but the ecodormancy may receive influence from the rootstocks.
2. Hormones were extracted from dormant buds of the Gala and Castel cultivars grafted on Maruba, Maruba/M9 and M9 rootstocks. Zeatin (Z), gibberellins (GA<sub>3</sub> and GA<sub>4</sub>), jasmonate (JA), indole-acetic acid (IAA), abscisic acid (ABA), and salicylic acid (SA) were quantified by UPLC-MS/MS.
3. The hormones are present from the middle of endodormancy onward. ABA acts as the hormonal repression of meristem until it reaches full cold accumulation, when GA becomes present. Therefore, GA is acting in growth together with Z, and IAA with JA near the budbreak. 'Castel' and 'Gala' present diverse chilling requirements but showed similar hormonal balance. Vigorous (Maruba) and dwarfing (M9) rootstocks showed different budbreak time and diverged in ABA quantification in endo-to-ecodormancy.
4. Quantification confirmed ABA and GA as the main dormancy regulators. M9 rootstock maintains bud dormancy through ABA regulation, in contrast, the Maruba rootstock does not delay the budbreak.

Key words: apple, dormancy, *Malus x domestica*, hormone quantification, rootstocks



## 1. INTRODUCTION

Temperate tree species historically face large seasonal variations especially in environmental factors such as temperature, day length and water availability. In response to this climatic context, trees developed some protective strategies including bud dormancy. This physiological process is classified in endodormancy, ecodormancy and paradormancy. The first is the inability to grow in favorable conditions, the second is the growth inhibited by unfavorable conditions and the third is the growth inhibition by other part of the plant (apical dominance; Rohde & Bhalerao, 2007). Therefore, bud dormancy is directly influenced by environmental changes and can be modulated differently among genotypes. During endodormancy in autumn, leaves fall and apical meristems are closed inside scales, forming the terminal buds. During this process there is also the accumulation of callose in plasmodesmata to avoid water influx and to protect bud cells against low temperatures and drought (Arora *et al.*, 2003; Rinne *et al.*, 2011). To overcome this stage, usually chilling accumulation and/or light length changes are required, but specifically for *Rosaceae* family the chilling accumulation is the main environmental factor to release the endodormancy (Heide & Prestrud, 2005; Heide, 2008). The chilling requirement (CR) is very variable among species and cultivars, being genetic factors the main responsible to determine this characteristic (Lang *et al.*, 1987; Falavigna *et al.*, 2014). Once CR is achieved, the plant passes to the ecodormancy stage and remains dormant during the winter and the initial part of spring until the environmental conditions become favorable to growth (Lang *et al.*, 1987).

Apple (*Malus × domestica* Borkh.) is an economically important tree (Hummer & Janick, 2009) with defined growth and dormancy phases (Labuschagné *et al.*, 2002). The CR is measured in chilling units (CUs) or chilling hours (CHs) in apple trees and it is defined as one hour at temperatures below 7.2 °C (Chandler, 1937). This low temperature requirement is a problem for farmers of *Rosaceae* plants since they have to choose the right cultivar to the right region (Atkinson *et al.*, 2013). In a context of climatic changes where winter chilling is becoming less available, the choosing of right cultivar is becoming more decisive to keep the sustainable production and avoiding the

use of chemicals to promote budbreak (BB) (Hänninen & Tanino, 2011; Legave *et al.*, 2015). Therefore, for the selection of adapted genotypes to warmer winters becomes necessary to advance knowledge about the metabolism of bud dormancy. Despite the recent advances, there is still a lack of information, especially about the hormonal balance during the endodormancy period.

Recent studies highlight the differential expression of genes from hormone-controlled pathways, during the transition from endo- to ecodormancy, as well as during the dormancy release steps or budbreak (BB) in the *Rosaceae* family (Falavigna *et al.*, 2014; Porto *et al.*, 2015; Kumar *et al.*, 2017; Tuan *et al.*, 2017). In peach, for example, ABA presents a decrease from dormancy set to BB (Wang *et al.*, 2016) while, in pear, a stable quantity of ABA was observed from dormancy set until endo-ecodormancy transition, in which occurs an ABA peak and then a drop until BB (Tuan *et al.*, 2017). GA has already been reported as an essential factor for callose degradation in the plasmodesmata during BB, once it is a  $\beta$ -1,3-glucanase inductor (Rinne *et al.*, 2011). GA is also the major regulator of BB in cherry (Zhu *et al.*, 2015) and it was reported the differential expression of genes for GA synthesis during BB in apple (Kumar *et al.*, 2017) and between apple cultivars with diverse CR (Falavigna *et al.*, 2014). Other hormones like Z and IAA have already been associated as inter regulators (Müller & Leyser, 2011) with differential gene expression related to their pathways in apple dormancy (Falavigna *et al.*, 2015; Porto *et al.*, 2015, 2016; Kumar *et al.*, 2017) and in BB of apple (Kumar *et al.*, 2017), but with inconsistencies when compared with pear (Tuan *et al.*, 2017), and peach (Leida *et al.*, 2012; Wang *et al.*, 2016).

Despite the contrasts of tissues, there is a strong correlation between the dormancy of bud meristem and the dormancy of embryo seed mechanisms. Most considerations on gene expression regulation and suppositions of hormone homeostasis in bud dormancy were based on data from seed dormancy (Leida *et al.*, 2012; Debska *et al.*, 2013; Wang *et al.*, 2016). All these data proposed that GA and ABA are the most probable regulators in apple, pear and peach, as well as in other plants that exhibit winter dormancy (Rinne *et al.*, 2011; Wang *et al.*, 2016; Kumar *et al.*, 2017). This mechanism is based on a relationship between the quantity of ABA and GA, in an ABA-GA balance.

In order to facilitate the practices of management and improve the quality of production, apple growers use rootstocks in their orchards. In this way, different rootstock and scion combinations provide variations in growth vigor, plant size and production rate (Hooijdonk *et al.*, 2011). Although the roots are sources of hormones, information on how the rootstocks can modulate the hormonal balance in apple buds as well as in the evolution of the state of dormancy is not yet available.

Since dormancy is a vital phase in plant life and production, the comprehension of its mechanisms and influences are fundamental. Therefore, the present work seeks an advance in the bud metabolism with precise quantification of six hormonal classes (ABA, GA, IAA, JA, SA, Z) during apple tree dormancy. As a strategy to understand that, it was compared the contrasts between two apple cultivars with different chilling requirement as scion. These scions were grafted on three different commercial rootstocks to monitor the influence of different vigor from rootstock in hormonal balance of the buds during the dormancy period. It was also evaluated the influence of the rootstocks on phenology and on the hormonal balance of the buds during dormancy.

## 2. MATERIAL AND METHODS

### Plant Material

Plant material was obtained from an orchard field located at EMBRAPA's Experimental Station, in Vacaria, RS, Brazil (28°51'39"87S, 50°88'12"58W, 971 m altitude). The experimental orchard was implanted in 2009 and was composed of three randomized blocks, where treatments were composed of the combination of two apple cultivars as scions, Gala Standard (Gala) or Castel Gala (Castel), and three rootstocks, Marubakaido, M9 or Marubakaido with M9 as inter-stock). Each experimental unit, considering the blocks and scion/rootstock combination, was formed by five plants. Considering the sum of cold accumulated in the field, the samples of buds in these trees were collected during the winter of 2016. In each date, 40 terminal already dormant buds

completely closed [phenological classification A, according to the Fleckinger scale, (EPPO, 1984)], for hormone quantification, and 22 twigs with 35 cm, for phenological analyses, were collected in each experimental unit. The samplings were always carried out in the same period of the day, between 10 and 12 h, with buds destined to hormonal analysis being frozen directly in liquid nitrogen and twigs wrapped in moist paper inside plastic bags.

### Phenology Analysis

The 22 twigs from each scion/rootstock combination were surface decontaminated for 15 min in 2.5% sodium hypochloride, followed by 3 washes in autoclaved water. Twigs were conditioned in a commercial phenolic foam right after base tips were chopped, and put inside a growth chamber under BudBreak forcing conditions ( $25 \pm 1.5^{\circ}\text{C}$ , 16 h light photoperiod,  $80\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , 75% relative humidity). The BB percentages were determined from the total number of terminal buds showing green tips up to 45 days of treatment. For BB progression analysis, the BB rate was verified every day until the 45<sup>th</sup> day.

### Hormone Quantification

Hormone quantification was performed as described in Chapter 1. Forty dormant buds of each scion/rootstock/block combination were ground in a cryogenic grinder (SPEX<sup>®</sup> SamplePrep, model 6870D Freezer/Mill<sup>®</sup>) and kept at  $-20^{\circ}\text{C}$  until extraction. From the milled samples, 500 mg were employed for hormone extraction with 4 mL extraction solution (methanol:water:formic acid, 75:20:5, v:v:v) with 2 mM citric acid and kept at  $-20^{\circ}\text{C}$  for 3 h. After that, the samples were extracted for 25 min at  $4^{\circ}\text{C}$  in an ultrasound bath (40kHz frequency), followed by centrifugation at  $1,750 \times g$  for 30 min at  $4^{\circ}\text{C}$ . The pellet was re-extracted twice with 3 mL of extraction solvent, kept for 6 h (second extraction) and 12 h (third extraction) at  $-20^{\circ}\text{C}$  and sonicated at the same condition described above. After the last centrifugation, supernatants were combined, dried in a vacuum centrifuge ( $40^{\circ}\text{C}$ ) and suspended in 1 mL of water. The diluted samples were applied on Oasis MCX cartridges (150 mg Sorbent, Water Corp., USA), which were previously wetted with 5 mL of

methanol and conditioned with 5 mL of water. The loaded cartridge was washed with 3 mL of 1 M formic acid, and sequentially eluted with 4 mL of methanol, 1 mL of water and three ammonium hydroxide elutions [1.5 mL at 0.004 M, 1.5 mL at 0.04 M and 2 mL at 0.4 M, all in methanol:water (4:1, v:v)]. The eluted solutions were combined (~10 mL per sample), dried in a vacuum centrifuge (40 °C), suspended in 75 µL of methanol and filtered through a 0.22 µm PVDF filter. The quantification analysis was carried in UPLC-ESI-MS/MS (Water Corp., USA). For each scion/rootstock combination, a duplicated extraction was performed with the addition of an internal standard to verify the recovery efficiency and the matrix effect (ME) during the extraction and quantification steps. Results were expressed in nanograms of hormone per grams of fresh weight sample (ng/g).

#### Data Analysis

Statistical analysis was performed in GraphPad Prism v6 using Two-way ANOVA repeated measures followed by Tukey test for parametric samples. Non parametric samples were analyzed by Friedman test and One-way ANOVA followed by Geisser-Greenhouse's epsilon.

### 3. Results

The historical chilling accumulation (< 7.2°C) in the experimental field in Southern Brazil from April to September is 601 CH. In same period of 2016, the overall chilling accumulation was 901 CH according to the Inmet's weather station located beside the experimental field. This relative high availability of cold allowed us to investigate the hormonal balance of both cultivars in their 'natural' progression of dormancy in the orchard, with the chilling requirement fully attended in each genotype. The time points evaluated are shown in Table 1.

*Table 1 Chilling hours (CH) accumulated by apple dormant buds and dates of harvesting.*

| Date       | Hours below 7.2 °C |
|------------|--------------------|
| 05/04/2016 | 118                |
| 06/09/2016 | 325                |
| 06/23/2016 | 496                |
| 07/28/2016 | 707                |
| 08/18/2016 | 778                |

### **Phenology Analysis**

The phenological analysis of ‘Castel’ twigs showed a gradual increase in dormancy overcome (BB) as the sum of CH approached the demand of this cultivar on the 3 rootstocks analyzed (Maruba, M9 and MaM9). All rootstock combinations started with BB under 18% at 118 CH and remained low until 496 CH for Maruba and 707 CH for M9 and MaM9. At 707 CH, all ‘Castel’ plants were in ecodormancy (BB > 50%) and in 778 CH they were in full flower bloom in the field, with some cases of fruit set (Figure 13 a). Gala did not perform a progressive BB during the period of analysis under forcing conditions, staying in endodormancy state (BB < 50%) (Figure 13 b). In the same way, ‘Gala’ just reached the bud burst after accumulation of 800 CH in the field.

As ‘Castel’ plants presented differences in BB rates among the chilling points, it was evaluated the BB progression for each point. The rootstocks presented progressive increase in BB rates proportional to chilling accumulation, when in forcing conditions (Figure ). The twigs from ‘Castel’/Maruba plants achieve the highest rates of BB, with more than 70% at 496 CH (Figure a) with the fastest BB in 14 days. The difference was significant in BB rates at 325 CH when compared with 496 CH and 707 CH, but it was not significant when 496 CH and 707 CH were compared. The twigs from ‘Castel’/MaM9 plants presented a lower BB rate than the twigs from ‘Castel’/Maruba at 496 CH and 707 CH (Figure b). No statistic difference among MaM9 at 707 CH to Maruba or M9 was found (Figure 13 a). The twigs

from 'Castel'/M9 plants presented the lower rate of BB (Figure c), being significant when compared with 'Castel'/Maruba twigs, but not with Castel'/MaM9 (Figure 13 a).

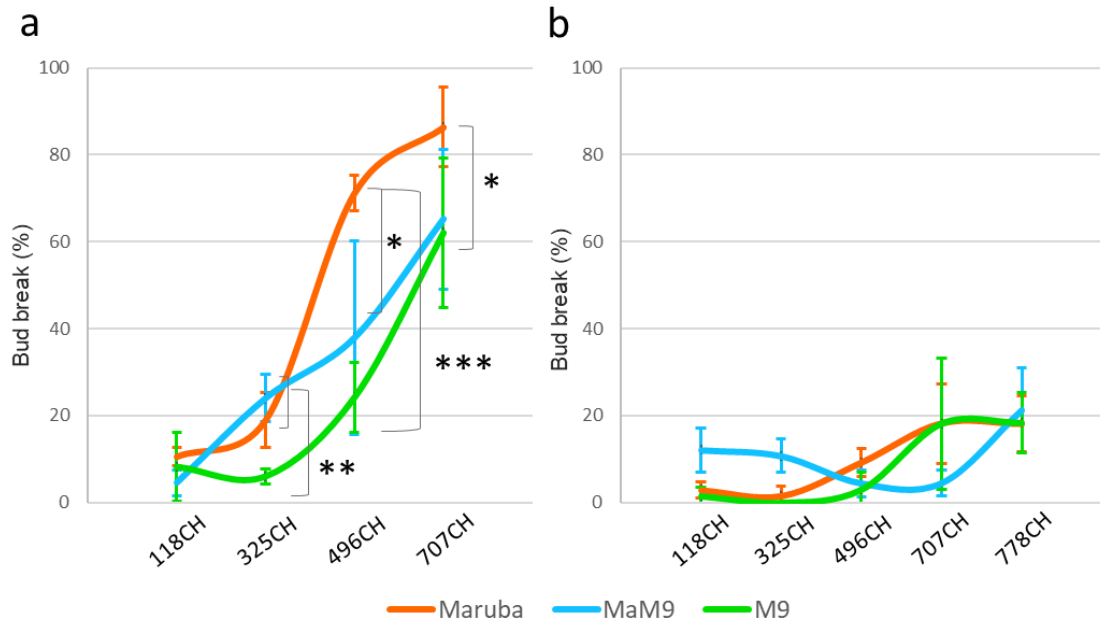


Figure 11 Comparison of Budbreak evolution in forcing conditions of twigs of 'Castel' (a) and 'Gala' (b) grafted on Maruba, MaM9 or M9 rootstocks, with field chilling accumulation in 2016. Error bars are representing the standard deviation (n=22). \* means  $p < 0.05$ ; \*\* means  $p < 0.01$ ; \*\*\* means  $p < 0.001$

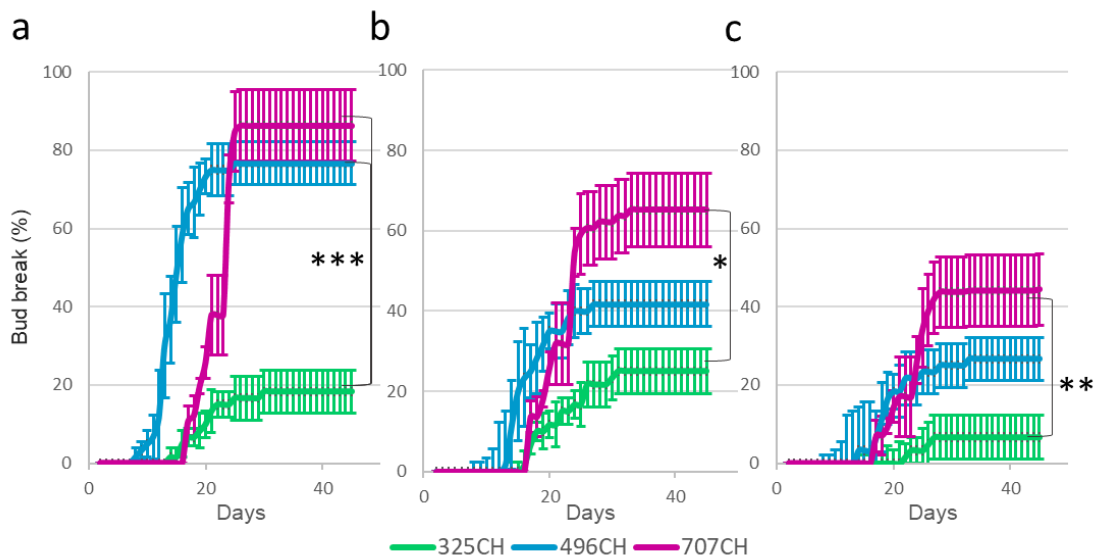


Figure 14 Comparison of budbreak evolution in forcing conditions of twigs of 'Castel' grafted on several rootstocks during dormancy in 2016 winter: a – Maruba; b – MaM9; c – M9. Error bars are representing the standard deviation (n=22). \* means  $p < 0.05$ ; \*\* means  $p < 0.01$ ; \*\*\* means  $p < 0.001$

## Hormonal Quantification in Bud Dormancy

The hormonal balance of buds in both cultivars was directly influenced by cold and rootstock. Six out of seven proposed phytohormones were quantified in the analysis. GA<sub>3</sub> achieved the LOD (limit of detection, with signal/noise > 3) but not the LOQ (limit of quantification, with signal/noise > 10) (AOAC, 2002; Matuszewski *et al.*, 2003), so this hormone was not considered in further analysis. In a default hormonal balance of ‘Gala’ and ‘Castel’, with proper chilling accumulation, the phytohormones behaved as shown in Figure 15.

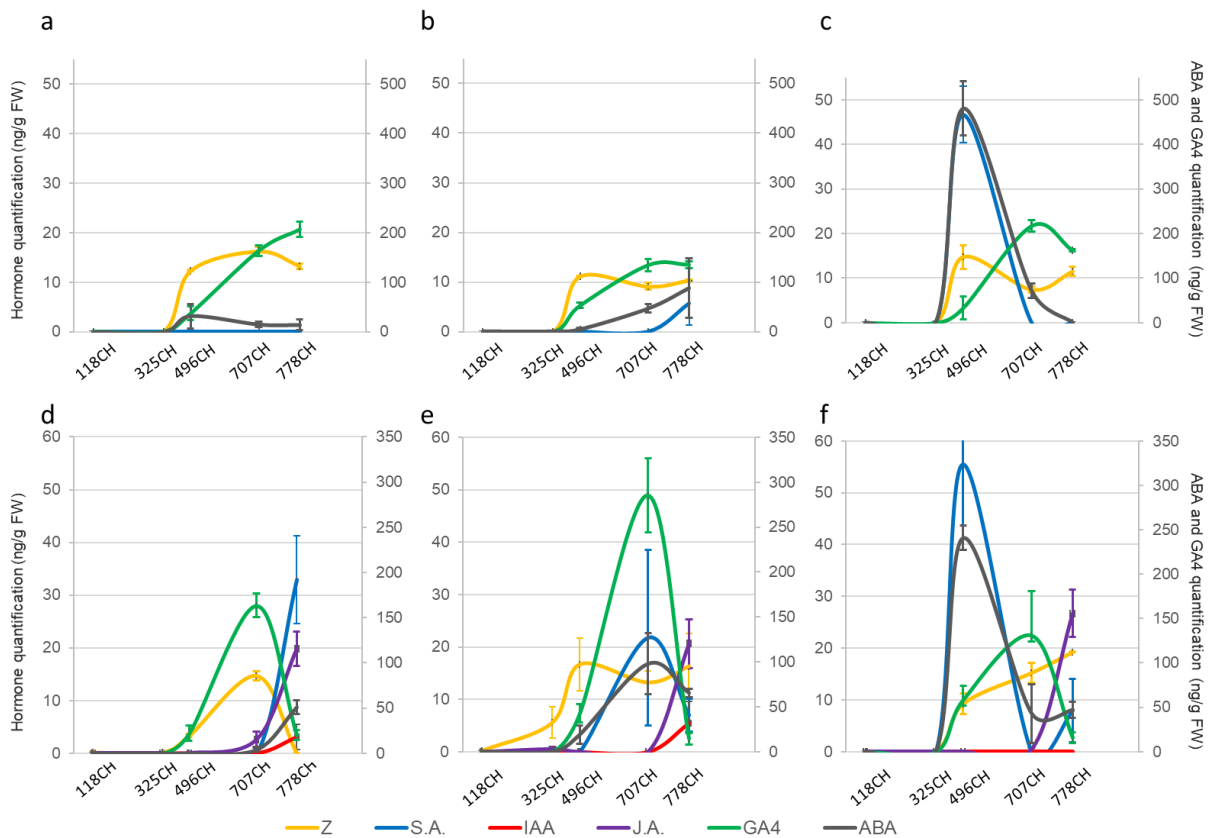


Figure 12 General summary of hormonal balance in 2016 winter of ‘Castel’ and ‘Gala’ with diverse rootstock combinations: A and D– Maruba; B and E – MaM9; C and F – M9. ‘Castel’ – D, E, F; ‘Gala’ – A, B, C. X axis are spaced by the number of days proportional to chill accumulated represented.

The ‘Castel’ and ‘Gala’ presented a similar hormonal balance until the 707 CH point. The hormone quantification was inexpressive at 118 CH and 325 CH. At 496 CH, ABA, SA, GA<sub>4</sub> and Z were present in different quantities



according to the rootstock. GA<sub>4</sub> presented a lower quantity at 496 CH, near to 50 ng.g<sup>-1</sup> FW in all cultivar/rootstock combinations. The GA<sub>4</sub> quantity rose to 130-200 ng.g<sup>-1</sup> FW at 707 CH, except in 'Castel'/MaM9 which achieved 275 ng.g<sup>-1</sup> FW, being it significantly higher than the others plants at this point. At 778 CH, the GA<sub>4</sub> quantification dropped to near zero for 'Castel' plants. For 'Gala' plants, the quantification at 778 CH was equal to that observed at 707 CH (Figure 16a and 4b).

ABA presented a statistically constant from 496 CH to 778 CH in 'Gala'/Maruba and 'Gala'/MaM9. For 'Castel'/Maruba, the ABA quantification was low until 707 CH and rose at 778 CH. In 'Castel'/MaM9, the ABA rose at 707 CH and kept the same quantity at 778 CH. For 'Gala'/M9 and 'Castel'/M9, the ABA presented the statistically significant highest peaks for each apple cultivar, with quantification dropping at 707 CH and 778 CH (Figure 16 c and d).

Z was absent at 325 CH in 'Castel'/MaM9 (Figure 17 b). At 496 CH, the Z quantification was near 13 ng.g<sup>-1</sup> FW for 'Gala' plants and 4-16 ng.g<sup>-1</sup> FW for 'Castel' plants. No statistically significant differences were observed for 'Castel' at 707 CH or 778 CH, except 'Castel'/Maruba, which was not quantified (LOQ < 3) at 778 CH (Figure 17 a and b).

SA presented a balance similar to ABA. 'Gala'/M9 and 'Castel'/M9 presented a statistically significant peak at 496 CH. SA was present at 707 CH in 'Castel'/MaM9 and at 778 CH in 'Castel'/Maruba, but neither were significant when compared to the other rootstocks (Figure 17 c and d).

JA and IAA were present detected and quantified only in 'Castel' plants. JA was present at 707 CH and rose to nearly 23 ng.g<sup>-1</sup> at 778 CH, without statistically significant differences among rootstocks (Figure 18 a). IAA was present only at 778 CH, with quantification near to 4 ng.g<sup>-1</sup>. No statistically significant differences were observed (Figure 18 b).

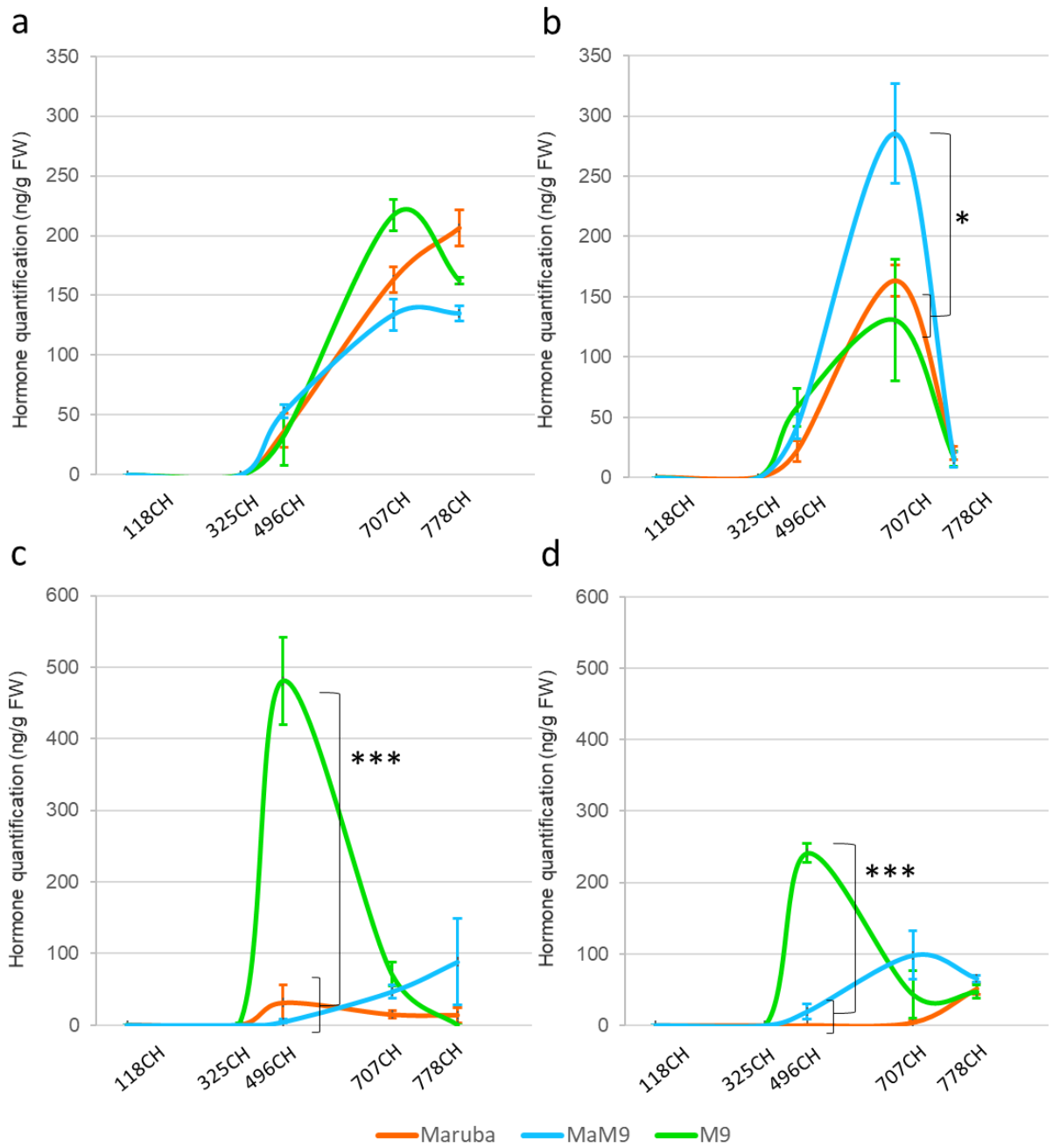


Figure 13 Comparison of hormone quantification among diverse rootstocks and cultivars of apple tree from 2016 winter. A and C – 'Gala'; B and D – 'Castel'. A and B – GA<sub>4</sub>; C and D – ABA. Error bars represent standard error. \* means  $p < 0.05$ ; \*\* means  $p < 0.01$ ; \*\*\* means  $p < 0.001$ .

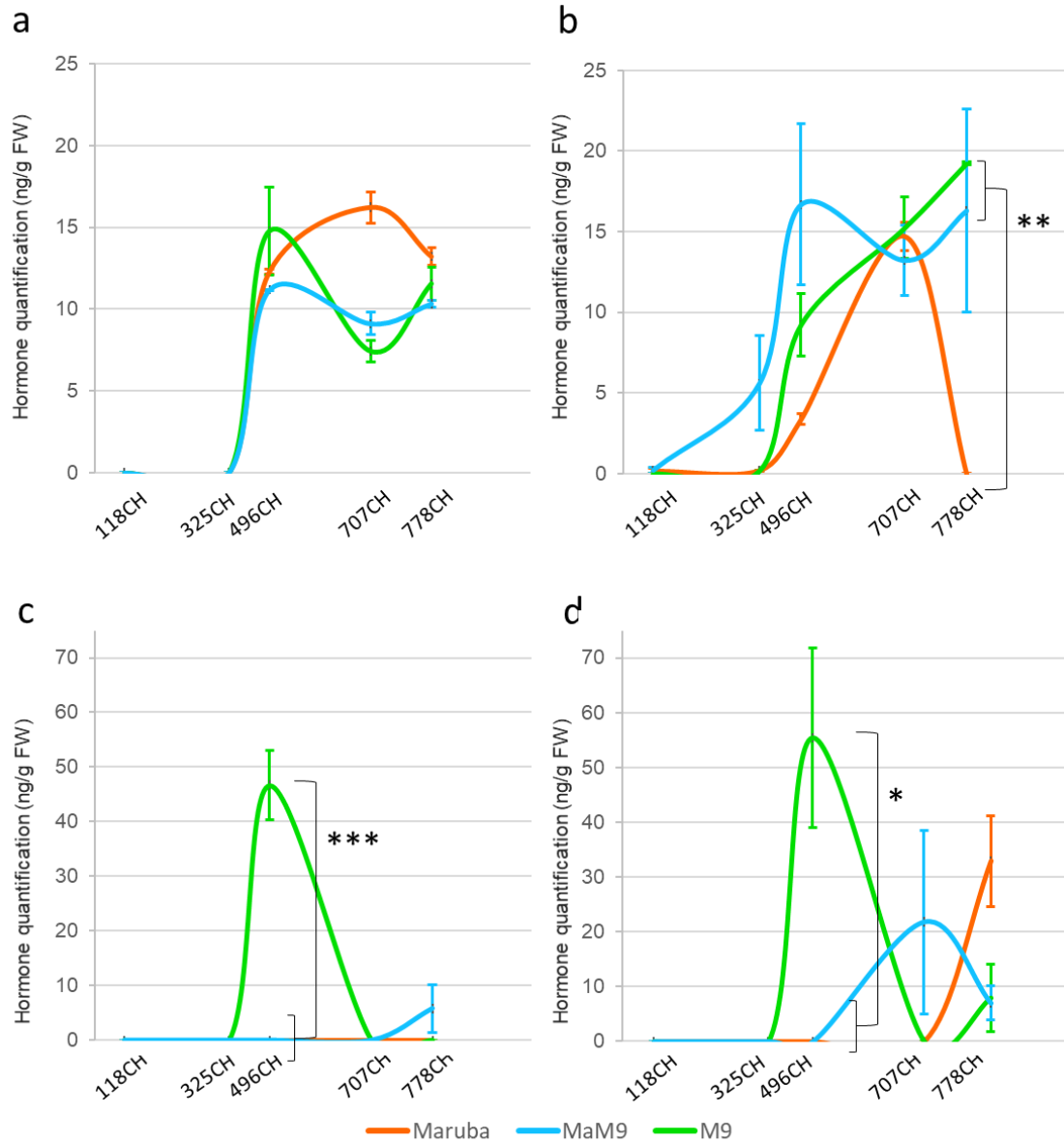


Figure 14 Comparison of hormone quantification among diverse rootstocks and cultivars of apple tree from HC winter (2016). A and C – ‘Gala’; B and D – ‘Castel’. A and B – Z; C and D – SA. Error bars represent standard error. \* means  $p < 0.05$ ; \*\* means  $p < 0.01$ ; \*\*\* means  $p < 0.001$ .

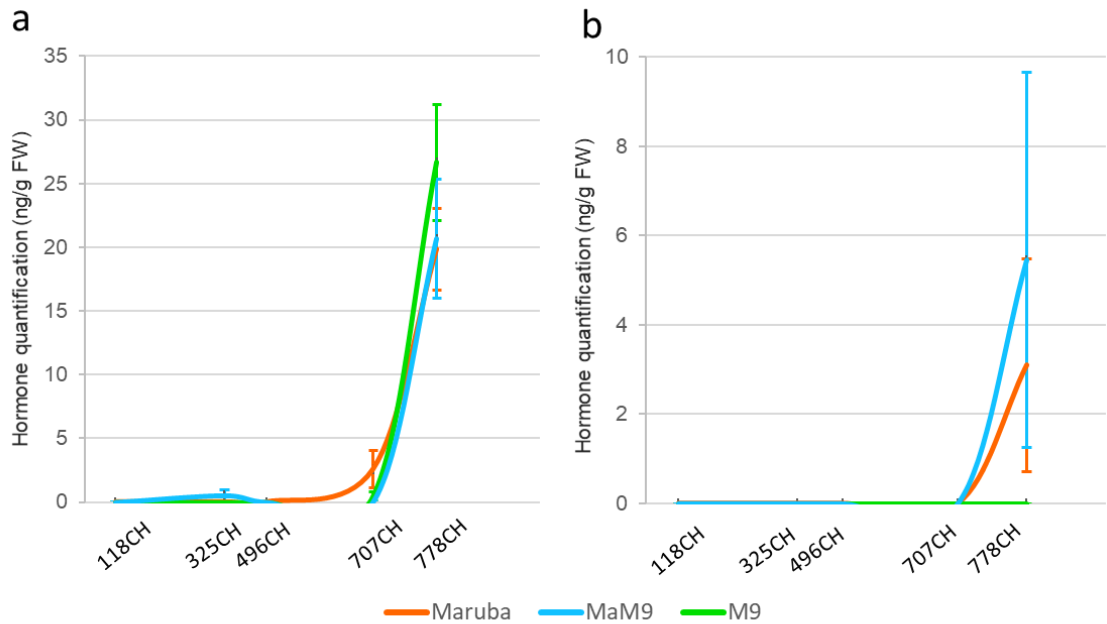


Figure 15 Comparison of hormone quantification of 'Castel' among diverse rootstock from 2016 winter. A – JA; B – IAA.

#### 4. Discussion

##### Phenological Analysis

The result of phenological analysis demonstrated that the BB in 2016 occurred after the expected chilling acquirement. Our results showed that 'Castel', that commonly needs to accumulate nearly 330 CH to properly BB (Anzanello *et al.*, 2014), had its BB retarded with M9 or MaM9 rootstock, occurring only after 496 CH under forcing conditions (Figure 13 and Figure ). The data obtained in the current study for 'Castel'/Maruba fits the data previously described for 'Castel'/M9 (Anzanello *et al.*, 2014). The divergences in chilling accumulation to BB could be explained by the differences in places where the samples were harvested, recent local climatic history, plant age and other factors. It was already reported differences in time to BB in apple trees planted in different places and thermal regimes of the globe (Legave *et al.*, 2015). The M9 rootstock with 'Castel' presented lower BB rates and later than the vigorous rootstock Maruba, which is compatible with literature, that describes M9 as a BB retarder in apple tree (Tworkoski & Fazio, 2016). 'Gala'

showed the same BB delay under forcing conditions for all rootstocks, with BB occurring only in the field with more than 800 CH. Literature describes 'Gala' as needing 600 CH in south Brazilian conditions (Anzanello *et al.*, 2014). This suggests that the lack of chilling requirement in previous years could have affected the chilling counting or chilling sensibility of buds in the next winter. The influence of warming waves during dormancy and flowering delay in the same cycle/year have already been reported (Anzanello *et al.*, 2014; Legave *et al.*, 2015). A previous study showed that no significant differences in growth and BB time in one cycle/year can accumulate as the tree grows and it may become significant in a long-term analysis (Tworkoski & Fazio, 2016). This reinforces the idea of continuity and stacking of reactions to environmental signals, possibly explain this delay of BB in high chilling (2016). A study described glucose and sucrose demand as an important growth drive as well as others known hormones, once the stem is consuming sugar, it would inhibit the other stems (Mason *et al.*, 2014). 'Gala' presented great amount of lateral buds in the twigs collected (data no shown), which could have drained force/glucose to BB and inhibited the terminal/apical bud which were already delayed to BB. Since the twig is separated from root, the sugar content is limited. If the lateral buds were considered in phenological test, the 'Gala' twigs achieved > 50% BB at 707 CH, in the same period that 'Castel' terminal buds achieve that stage. A hypothesis suggests that 'Gala' enters in a deeper bud dormancy than 'Castel' (Anzanello *et al.*, 2014). Based on that, it is possible that 'Gala' is more sensitive than 'Castel' to those interfering conditions from previous years, especially warmer winters.

### **Hormone Balance during the Dormancy of Apple Trees**

From the seven proposed phytohormones to be analyzed, six were quantified. GA<sub>3</sub> achieved the LOD but not LOQ. It seems that GA<sub>3</sub> possibly does not perform an important role during dormancy in apple tree buds and GA<sub>4</sub> would be the one of the principal gibberellin form in apple dormancy as in hybrid aspen dormancy (Rinne *et al.*, 2011) and tobacco seeds (Arora *et al.*, 2003).

The general hormone homeostasis during apple tree dormancy in the 2016 winter was intriguingly similar between cultivars, even with very distinct chilling requirements and different rootstocks. There was a low overall hormone quantification in the first two points evaluated, 118 CH and 325 CH. Possibly due to action of other hormonal forms or metabolisms actors. ABA quantification in pear buds showed the same pattern that in this work, with a peak right before endo- to ecodormancy transition (Tuan *et al.*, 2017). Apple and pear dormant buds present similar dormancy induction and endodormancy transcription results (Heide & Prestrud, 2005), which corroborates ABA quantification result.

A previous work that quantified ABA in dormant buds and seeds in peach (Leida *et al.*, 2012; Wang *et al.*, 2016), demonstrated ABA with high amounts at dormancy entrance but a progression decrease until no detection was possible in the endo- to ecodormancy transition. The differences in ABA amounts found during endodormancy of apple and pear buds compared to peach buds could be explained by the differences in ABA quantification methods. In this and in the pear work, the quantification was done by UPLC-ESI-MS/MS and by Elisa in the peach work. Elisa quantification could count conjugated multiforms of ABA while in pear and in this work it was measured a single form of metabolically active ABA. The differences between quantification methods and results could suggest that the endodormancy phase (118 CH and 325 CH) could be ABA rich but not active in apple and pear bud dormancy.

The differences in ABA modulation among rootstocks were significant only at 496 CH point, in which the plants on M9 rootstock presented the greatest ABA peak in both apple cultivars. This agrees with the analyzes of xylem sap in M9 rootstock which present higher ABA concentration than other more vigorous rootstocks in apple tree (Yadava & Lockard, 1977; Kamboj *et al.*, 1999; Tworkoski & Fazio, 2016). This opposite relation of rootstock vigor and ABA concentration is also found in tomato rootstock (Martínez-Andújar *et al.*, 2016). In 'Castel' plants, 'Castel'/M9 had the highest ABA quantification at 496 CH, with lower BB rates. The 'Castel'/Maruba had no ABA quantification at 496 CH and the highest BB rates, being in ecodormancy from this point. These results indicate a link between ABA at this CH point and the endodormancy release in 'Castel' plants. Callose barrier is defined as a divisor in bud endo-

ecodormancy in physiology and in seed dormancy (Arora *et al.*, 2003; Rinne *et al.*, 2011). As 'Castel'/Maruba passed to ecodormancy at 496 CH, it is possible to assume that the communication between the bud and the plant is functional. Applying this connection concept to other 'Castel' plants, we can hypothesize that the ABA quantified from 496 CH onwards is from the rootstock.

'Gala' plants presented the same ABA pattern of 'Castel', but with different phenology, which suggest another BB repressor mechanism in 'Gala'. A known repressor mechanism in bud dormancy in *Rosaceae* is from *DORMANCY-ASSOCIATED MADS-box (DAM)* genes. They were discovered in the *evergrowing* peach mutant, which is unable to enter dormancy (Bielenberg *et al.*, 2008). It was showed that 'Gala' presents greater quantity for a longer time of expression of *DAM* genes than 'Castel' (Porto *et al.*, 2016), which could explain this difference in BB rates even with similar hormonal profile.

GA<sub>4</sub> presented the same patterns of accumulation in all rootstock and cultivar combinations, except for 'Castel'/MaM9. The GA<sub>4</sub> was present since 496 CH, at low level, and then presented a peak at 707 CH ('Castel') and 707 CH-778 CH ('Gala'). At 496 CH, the GA<sub>4</sub> appears to be related to  $\beta$ -1,3-glucanase activation in the callose release process (Rinne *et al.*, 2011), which support the idea of free communication from 496 CH onward, mentioned earlier. The GA presence at 707 CH and 778 CH points appear to be related to the development and expansion of the tissues inside the bud, as in seed and bud dormancy of peach (Leida *et al.*, 2012) and other seeds (Kucera *et al.*, 2005). The equality in GA levels and expression modulation (Kumar *et al.*, 2017) indicates that the GA is synthesized in the bud, showing independence from the rootstocks.

The ABA and GA present an opposite behavior during the evolution of bud dormancy, except for MaM9 plants. This difference in MaM9 plants is possibly related to their double graft (M9/Maruba and then Cultivar/M9/Maruba), in which this M9 piece changes the communication structures, and influences the hormonal signaling. This ABA/GA balance in maintaining and release of dormancy is already well described and widely found in seed dormancy (Leida *et al.*, 2012; Debska *et al.*, 2013; Liu *et al.*, 2015; Wang *et al.*, 2016). This mechanism is complex and presents inner steps in other metabolic pathways,

as oxidative process (Considine & Foyer, 2014), which suggest that the bud dormancy in apple tree could present the same behavior.

SA presented the same pattern as ABA, with the highest peak at 496 CH in M9 grafted plants. SA is reported to be associated with ABA- and GA-related pathways, acting as a 'rheostat' and contributing to both hormones (Rivas-San Vicente & Plasencia, 2011). SA has been associated with biotic and abiotic stresses, but little is known about its role in growth, development and especially in bud dormancy. Based on the closest mechanism, seed dormancy and germination, exogenous SA presented a positive interaction with GA/germination and a negative interaction with ABA when in abiotic stress, but the opposite was observed under normal conditions (Rajjou *et al.*, 2006). Therefore, it is not yet possible to define the role of SA in dormancy, even though it clearly shows a relationship with ABA and, consequently, BB delay in M9 plants.

At 496 CH, Z became detectable and it indicated some metabolic activity, possibly signaling a division process (Kieber & Schaller, 2014) and/or drain force of the bud. In addition, the presence of Z may also indicate the reconnection of buds with the plant, since roots and node tissues near the meristem are important sources of cytokinins (Müller & Leyser, 2011). The general behavior of Z levels is an almost constant presence at the 8-16 ng.g<sup>-1</sup> range for 'Gala' from 496 CH until 778 CH, and a rising quantification at the 4-15 ng.g<sup>-1</sup> range from 496 CH to 778 CH in 'Castel'. Despite the differences in Z modulation among rootstocks, they did not differ statistically. It is interesting to highlight the fact that 'Castel'/MaM9 presented a precocious Z presence at 325 CH and, at 707 CH, it presented the highest GA<sub>4</sub> quantification, which suggests a connection between these hormones in dormancy, despite an evidence of independence of these events in *A. thaliana* flowering (Greenboim-Wainberg *et al.*, 2005). A previous work with the measure of Z at the sap of shoot and root in apple trees with different rootstocks demonstrated that more vigorous rootstocks like M106 presented higher amounts of general cytokinin and greater Z proportion than dwarf rootstocks like M9 and M27 (Kamboj *et al.*, 1999). On the other hand, another work with xylem sap from 'Gala' on M7, M9, M27 or M111 rootstocks showed no difference in Z profile among the rootstocks



(Hooijdonk *et al.*, 2011), what was similar in scion/rootstock combination and results presented in this work. Since there is plenty of cytokinin versions, the rootstocks could influence the bud with others forms of this class of hormones than Z. Both 'Castel' and 'Gala' presented similar Z patterns but the rootstock influence on Z quantities was not consistent in both cultivars. Despite the difference in phenology among rootstocks, the equality in Z quantity suggests that Z does not influence BB directly in an adequate chilling acquisition.

Finally, at 778 CH, 'Castel' plants reached BB in the field and this was also reflected in the hormone profile with an increase in IAA and JA, a reduction of GA<sub>4</sub> and a minor presence of ABA as well. These hormonal modulations are fully compatible with recently published data of Kumar *et al.* (2017) showing the role of ABA in flowering with other known hormones. 'Castel' plants presented the same JA pattern, independently of rootstock. JA is related to flower development and fertility, as reviewed by Devoto & Turner (2005). This indicates that the role of JA is strictly related to flowering and is produced in the tissues of buds. IAA presented a similar behavior, but in lower concentrations. Even though 'Castel'/M9 had no IAA quantification, it is statistically equal to 'Castel'/MaM9 and 'Castel'/M9. As a hormone of apical dominance (Wilkinson & Davies, 2010), the presence of IAA at this point, when the bud is opening, is clearly related to dominance of apical meristems.

At 778 CH, 'Gala' keeps the same hormone proportion that it was observed at 707 CH, showing that 'Gala' is still in the same phase (of dormancy) while 'Castel' passed already to active growth. The GA<sub>4</sub> quantification presented a significant difference between cultivars at 778 CH point. This difference was probably due to the fact that 'Castel' presented BB in the field at this point, with the development of leaves and flowers. Possibly, this is the main difference in time to overcome bud dormancy, with Castel progressing faster the GA<sub>4</sub> wave (ecodormancy) and Gala slowly progressing this stage (possibly still in endodormancy).

Summarizing the hormonal balance during bud dormancy progress, there is the endodormancy trigger with the first 100 CH for 'Gala' and 70 CH for 'Castel' (Anzanello *et al.*, 2014). This trigger decreases the metabolic activity of

phytohormones possibly due to repressor genes until 496 CH. The main regulators in apple tree dormancy are ABA and GA with antagonist balance for both 'Gala' and 'Castel' independent of rootstocks. ABA, SA and Z rise at 496 CH. After enough CH accumulation to transit from endo- to ecodormancy, the fall of ABA occurs and GA<sub>4</sub> rises. Physiologically speaking, it could mean the dismantlement of the callose barrier by GA<sub>4</sub> induced  $\beta$ -1,3-glucanases around 496 CH, allowing Z entrance in the bud. This event allows the bud to get access to water, carbohydrates and cytokinins from roots. After that, ABA falls drastically, followed by the drop of SA, and the rise of GA<sub>4</sub> and Z at 707 CH, indicating a metabolism unlock and preparing to BB in the field when environment becomes conducive. Until this point (707 CH), 'Castel' and 'Gala' cultivars present a very similar hormonal profile. However, at 778 CH, the bud of 'Castel' is totally ready to open and grow, with the rise of JA, Z and ABA, with the first one related to flower formation, and the Z with cell division and tissue expansions, and the ABA possibly with the abscission of parts that are not more necessary such as the scales and/or rootstock communication. In another way, for 'Gala' plants, the 778 CH time presented the same pattern of that at the 707 CH point, showing that the terminal buds of 'Gala' are still in the same stage (dormancy), while 'Castel' plants have passed to the growing stage (Figure 19).

For 'Castel', which presents half of the chilling requirement of 'Gala', the ABA peak at 496 CH is relatively shorter than in 'Gala', with the same GA<sub>4</sub> peak at 707 CH. This is accompanied by the reduction in oxidized glutathione (Santos *et al.*, 2011). Therefore, if we use the oxidative bridge among ABA and GA through redox regulation (Considine & Foyer, 2014), we can infer that 'Castel' does not reach a *deep dormancy*, as the one observed in 'Gala'.

Rootstocks showed few but important differences among them, especially in BB rates during forcing conditions, what infers that they could respond faster to some condition changes. Under forcing conditions, 'Castel'/Maruba showed faster BB than other rootstocks at 496 CH. At the same time, 'Castel'/Maruba did not show the ABA peak at 496 CH, which must be the cause of this earlier transitions to ecodormancy. This also explains why 'Castel'/M9 showed minor BB rates and the delay in BB rise at 707 CH, with higher ABA quantification at 496 CH. Maruba has a larger root system, being a

vigorous rootstock (Fioravanço *et al.*, 2016), and it could be explained by a lack of ABA action in the dormancy sustain or an excess of growth promoters (GA and Z possibly). In another way, M9 shows higher repressor conditions, delaying BB and providing a minor ratio growth/repression. The dwarfing effect of M9, in part due to reports of its disorganized vascular system (Tworkoski & Fazio, 2011, 2016), may be restricting bud supply in water, carbohydrates and hormones properly, with lower IAA, GA and CK fluxes and excess of ABA accumulation. The MaM9 showed an intermediate response to environmental cold effect among rootstocks, with high similarity to Maruba. Interestingly, this rootstock combination showed a higher GA<sub>4</sub>/ABA ratio and relative higher presence of Z, which reveals a role in BB induction (for 'Castel') not only in ABA decrease or GA<sub>4</sub> accumulation. This also could explain about the more diffuse architecture and more controlled scion growth under its control (Seleznyova *et al.*, 2003).

The difference among rootstocks may be summarized in ABA modulation, reinforcing its role as the major dormancy inducer (Kumar *et al.*, 2017). It is important to highlight that rootstocks do not impose the same effect on the different cultivars. An example is Z, which does not present equal rootstock influence on both cultivars. This feature was earlier reported in a work that comments the influence not only from rootstock on the scion, but also the influences from the scion on the rootstock (Tworkoski & Fazio, 2015). The difference may be small from one season to other one, but tends to accumulate and be more visible with season accumulations (Tworkoski & Fazio, 2011). Hence, these results should be taken as a long time inter-retro influence among cultivars and rootstocks.

Hormonal balance determines a critical role in dormant buds of apple trees. Endodormancy is apparently first controlled by other mechanisms than hormonal, most likely mediated by *DAM* genes, carbohydrates and proteins. Dormancy control by hormones occurs right before endodormancy release, with ABA acting as a metabolic repressor. Chilling accumulation induces GA synthesis and its accumulation may be related to callose barrier dismantlement. Z is part of meristem induction to budbreak with a constant presence, without significant differences among rootstocks. Other cytokinins may be more

decisive than Z in this period. SA seems to be a bud response (possibly collaborative) to high ABA quantities. IAA and JA do not seem to regulate dormancy, considering that they accumulate only at the time of budburst in the field, but may be acting in flower development. GA<sub>3</sub> does not appear to be part of dormancy regulation in apple buds, at least in quantifiable limits. The difference between apple cultivars with different chilling requirements was visible in the hormonal balance when employing the M9 rootstock, with 'Gala' presenting more ABA quantities than 'Castel'. Apple tree bud dormancy appears to be ruled by ABA-GA balance in a mechanism very similar to that described in seed dormancy. The rootstocks influence the scion BB mostly by contributing with ABA in the endo- to ecodormancy transition. The process of dormancy in apple is now clearer with the confirmation that ABA and GA play critical roles in dormancy as well as JA and IAA in flowering. This new knowledge creates new questions and opens perspectives to advances in the metabolic process of bud dormancy, which can aid in the selection of more adapted plants to the global warming climate state.

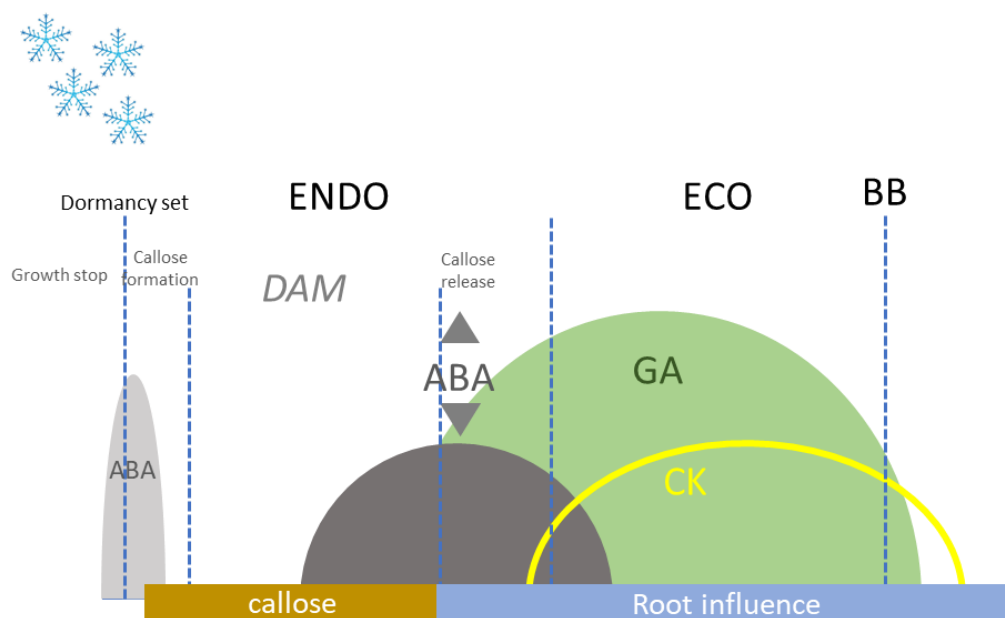


Figure 19: Proposal of a model for hormonal balance during bud dormancy in apple tree. The dormancy set is characterized by the presence of ABA in high amounts and formation of the callus barrier. Thereafter, there is no significant presence of quantified hormones in this work, but the presence of *DAM* genes in endodormancy has been reported. At the end of endodormancy, there is the

dismantling of the callose barrier and ABA can inflow from the roots. The amount of ABA is variable according to the rootstocks (indicated by the arrows). From the transition from endo- to ecodormancy, there is the presence of gibberellins and cytokinins as well, which are part of the development of ecodormancy. The influence of the roots (and rootstocks) occurs in the ecodormancy, being able to delay budding with ABA contribution.

### Acknowledgments

This work was supported by the Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Financiadora de Estudos e Projetos (FINEP).

### Authors' contributions

HPS and LFR conceived of and designed the experiments. JAG, DAS, HPS, performed the samplings in the field. JAG and DAS performed the hormonal extraction and HPS quantified the hormones. DAS made the phenological analysis. JAG, DAS, HPS, LFR and GP conceptualized the model and wrote the manuscript. All authors read and approved the final manuscript.

### References

- Anzanello R, Fialho FB, dos Santos HP, Bergamaschi H, Marodin GAB. 2014. Bud dormancy in apple trees after thermal fluctuations. *Pesquisa Agropecuaria Brasileira* **49**: 457–464.
- AOAC International Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis. 2002. Guidelines for Single Laboratory Validation (SLV) of Chemical Methods for Metals in Food.
- Arora R, Rowland LJ, Tanino K. 2003. Induction and release of bud dormancy in woody perennials: A science comes of age. *HortScience* **38**: 911–921.
- Atkinson CJ, Brennan RM, Jones HG. 2013. Declining chilling and its impact on temperate perennial crops. *Environmental and Experimental Botany* **91**: 48–62.
- Bielenberg DG, Wang Y, Li Z, Zhebentyayeva T, Fan S, Reighard GL, Scorza R, Abbott AG. 2008. Sequencing and annotation of the evergrowing locus in peach [*Prunus persica* (L.) Batsch] reveals a cluster of six MADS-box transcription factors as candidate genes for regulation of terminal bud formation. *Tree Genetics and Genomes* **4**: 495–507.
- Chandler WH. 1937. Chilling requirements for opening of buds on deciduous orchard trees and some other plants in California. *Bulletin* **611**: 3–11.
- Considine MJ, Foyer CH. 2014. Redox regulation of plant development. *Antioxidants & redox signaling* **21**: 1305–26.
- Debska K, Krasuska U, Budnicka K, Bogatek R, Gniadzowska A. 2013. Dormancy removal of apple seeds by cold stratification is associated with fluctuation in H<sub>2</sub>O<sub>2</sub>, NO production and protein carbonylation level. *Journal of Plant Physiology* **170**: 480–488.

- Devoto A, Turner JG. 2005.** Jasmonate-regulated Arabidopsis stress signalling network. *Physiologia Plantarum* **123**: 161–172.
- EPPO (1984). 1984.** *EPPO crop growth stage keys—apple and pear.*
- Falavigna V da S, Porto DD, Buffon V, Margis-Pinheiro M, Pasquali G, Revers LF. 2014.** Differential Transcriptional Profiles of Dormancy-Related Genes in Apple Buds. *Plant Molecular Biology Reporter* **32**: 796–813.
- Falavigna VS, Porto DD, Silveira CP, Revers LF. 2015.** Recent advances in genetics and molecular control of bud dormancy in pipfruits. *Advances in Plant Dormancy*: 107–122.
- Fioravanco JC, Czermainski ABC, Oliveira PRD de. 2016.** Yield efficiency for nine apple cultivars grafted on two rootstocks. *Ciência Rural* **46**: 1701–1706.
- Greenboim-Wainberg Y, Maymon I, Borochof R, Alvarez J, Olszewski N, Ori N, Eshed Y, Weiss D. 2005.** Cross talk between gibberellin and cytokinin: the Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. *the Plant Cell* **17**: 92–102.
- Hänninen H, Tanino K. 2011.** Tree seasonality in a warming climate. *Trends in Plant Science* **16**: 412–416.
- Heide OM. 2008.** Interaction of photoperiod and temperature in the control of growth and dormancy of Prunus species. *Scientia Horticulturae* **115**: 309–314.
- Heide OM, Prestrud AK. 2005.** Low temperature, but not photoperiod, controls growth cessation and dormancy induction and release in apple and pear. *Tree Physiology* **25**: 109–114.
- Hooijdonk B Van, Woolley D, Warrington I, Tustin S. 2011.** Rootstocks Modify Scion Architecture, Endogenous Hormones, and Root Growth of Newly Grafted ‘Royal Gala’ Apple Trees. *Journal of American Horticultural Science* **136**: 93–102.
- Hummer KE, Janick J. 2009.** Rosaceae: Taxonomy, Economic Importance, Genomics. In: Foltá KM, Gardiner SE, eds. *Genetics and Genomics of Rosaceae*. New York, NY: Springer New York, 1–17.
- Kamboj JS, Blake PS, Quinlan JD, Baker DA. 1999.** Identification and quantitation by GC-MS of zeatin and zeatin riboside in xylem sap from rootstock and scion of grafted apple trees. *Plant Growth Regulation* **28**: 199–205.
- Kieber JJ, Schaller GE. 2014.** Cytokinins. *The Arabidopsis Book* **12**: e0168.
- Kucera B, Cohn MA, Leubner-Metzger G. 2005.** Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**: 281–307.
- Kumar G, Gupta K, Pathania S, Swarnkar MK, Rattan UK, Singh G, Sharma RK, Singh AK. 2017.** Chilling Affects Phytohormone and Post-Embryonic Development Pathways during Bud Break and Fruit Set in Apple (*Malus domestica* Borkh.). *Scientific Reports* **7**: 42593.
- Labuschagné IF, Louw JH, Schmidt K, Sadie A. 2002.** Genetic Variation in Chilling Requirement in Apple Progeny. *Journal of the American Society for Horticultural Science* **127**: 663–672.
- Lang G, Early J, Martin G, Darnell R. 1987.** Endo-, para-, and ecodormancy: physiological terminology and classification for dormancy research. *HortScience* **22**: 371–377.
- Legave J-M, Guédon Y, Malagi G, El Yaacoubi A, Bonhomme M. 2015.** Differentiated Responses of Apple Tree Floral Phenology to Global Warming in Contrasting Climatic Regions. *Frontiers in plant science* **6**: 1054.
- Leida C, Conejero A, Arbona V, Gómez-Cadenas A, Liácer G, Badenes ML, Ríos G. 2012.** Chilling-dependent release of seed and bud dormancy in peach associates to common changes in gene expression. *PLoS ONE* **7**: 1–9.
- Liu Y, Müller K, El-Kassaby YA, Kermode AR. 2015.** Changes in hormone flux and signaling in white spruce (*Picea glauca*) seeds during the transition from dormancy to germination in

response to temperature cues. *BMC plant biology* **15**: 292.

**Martínez-Andújar C, Albacete A, Martínez-Pérez A, Pérez-Pérez JM, Asins MJ, Pérez-Alfocea F. 2016.** Root-to-Shoot Hormonal Communication in Contrasting Rootstocks Suggests an Important Role for the Ethylene Precursor Aminocyclopropane-1-carboxylic Acid in Mediating Plant Growth under Low-Potassium Nutrition in Tomato. *Frontiers in Plant Science* **7**: 1–16.

**Mason MG, Ross JJ, Babst BA, Wienclaw BN, Beveridge CA. 2014.** Sugar demand, not auxin, is the initial regulator of apical dominance. *Proceedings of the National Academy of Sciences* **111**: 6092–6097.

**Matuszewski BK, Constanzer ML, Chavez-Eng CM. 2003.** Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry* **75**: 3019–3030.

**Müller D, Leyser O. 2011.** Auxin, cytokinin and the control of shoot branching. *Annals of Botany* **107**: 1203–1212.

**Porto DD, Bruneau M, Perini P, Anzanello R, Renou JP, Dos Santos HP, Fialho FB, Revers LF. 2015.** Transcription profiling of the chilling requirement for bud break in apples: A putative role for FLC-like genes. *Journal of Experimental Botany* **66**: 2659–2672.

**Porto DD, Falavigna V da S, Arenhart RA, Perini P, Buffon V, Anzanello R, dos Santos HP, Fialho FB, de Oliveira PRD, Revers LF. 2016.** Structural genomics and transcriptional characterization of the Dormancy-Associated MADS-box genes during bud dormancy progression in apple. *Tree Genetics and Genomes* **12**: 15.

**Rajjou L, Belghazi M, Huguet R, Robin C, Moreau A, Job C, Job D. 2006.** Proteomic Investigation of the Effect of Salicylic Acid on Arabidopsis Seed Germination and Establishment of Early Defense Mechanisms. *Plant Physiology* **141**: 910–923.

**Rinne PLH, Welling A, Vahala J, Ripel L, Ruonala R, Kangasjärvi J, van der Schoot C. 2011.** Chilling of Dormant Buds Hyperinduces FLOWERING LOCUS T and Recruits GA-Inducible 1,3- $\beta$ -Glucanases to Reopen Signal Conduits and Release Dormancy in Populus. *The Plant Cell* **23**: 130–146.

**Rivas-San Vicente M, Plasencia J. 2011.** Salicylic acid beyond defence: Its role in plant growth and development. *Journal of Experimental Botany* **62**: 3321–3338.

**Rohde A, Bhalerao RP. 2007.** Plant dormancy in the perennial context. *Trends in Plant Science* **12**: 217–223.

**Santos HP, Revers LF, Leite GB, Bernardi J. 2011.** Caracterização da dormência hiberna em gemas de macieira. In: NACHTIGALL GR, ed. Inovações tecnológicas para o setor da maçã - Inovamaçã. Bento Gonçalves: Embrapa Uva e Vinho, 257–273.

**Seleznyova AN, Thorp TG, White M, Tustin S, Costes E. 2003.** Application of architectural analysis and AMAPmod methodology to study dwarfing phenomenon: The branch structure of 'Royal Gala' apple grafted on dwarfing and non-dwarfing rootstock/interstock combinations. *Annals of Botany* **91**: 665–672.

**Tuan PA, Bai S, Saito T, Ito A, Moriguchi T. 2017.** Dormancy-Associated MADS-Box (DAM) and the Abscisic Acid Pathway Regulate Pear Endodormancy Through a Feedback Mechanism. *Plant and Cell Physiology*.

**Tworcoski T, Fazio G. 2011.** Physiological and morphological effects of size-controlling rootstocks on 'Fuji' apple scions. *Acta Horticulturae* **903**: 865–872.

**Tworcoski T, Fazio G. 2015.** Effects of Size-Controlling Apple Rootstocks on Growth, Abscisic Acid, and Hydraulic Conductivity of Scion of Different Vigor. *International Journal of Fruit Science* **15**: 369–381.

**Tworcoski T, Fazio G. 2016.** Hormone and growth interactions of scions and size-controlling rootstocks of young apple trees. *Plant Growth Regulation* **78**: 105–119.

**Wang D, Gao Z, Du P, Xiao W, Tan Q, Chen X, Li L, Gao D. 2016.** Expression of ABA

Metabolism-Related Genes Suggests Similarities and Differences Between Seed Dormancy and Bud Dormancy of Peach (*Prunus persica*). *Frontiers in Plant Science* **6**: 1–17.

**Wilkinson S, Davies WJ. 2010.** Drought, ozone, ABA and ethylene: new insights from cell to plant to community. *Plant, Cell and Environment* **33**: 510–525.

**Yadava UL, Lockard RG. 1977.** Abscisic Acid and Gibberellin in Three Ungrafted Apple (*Malus sylvestris*) Rootstock Clones. *Physiologia Plantarum* **40**: 225–229.

**Zhu Y, Li Y, Xin D, Chen W, Shao X, Wang Y, Guo W. 2015.** RNA-Seq-based transcriptome analysis of dormant flower buds of Chinese cherry (*Prunus pseudocerasus*). *Gene* **555**: 362–376.



## 5 DISCUSSÃO

A dormência em gemas é um ponto importante da cultura da macieira e só tem sido melhor estudada recentemente (ARORA *et al.*, 2003; HEIDE & PRESTRUD, 2005; FALAVIGNA *et al.*, 2014; PORTO *et al.*, 2016). Muito do conhecimento desenvolvido foi baseado após a brotação das gemas, tanto no seu estudo agrônômico, bem como nos avanços moleculares e morfológicos (ARORA *et al.*, 2003; HEIDE & PRESTRUD, 2005; LEIDA *et al.*, 2010; SEIF EL-YAZAL *et al.*, 2014). Por ser pouco estudada, a gema em si ainda é um órgão relativamente desconhecido quando comparada com a literatura sobre sementes (LEIDA *et al.*, 2012; DEBSKA *et al.*, 2013; LIU *et al.*, 2015). No começo desse trabalho, focado na análise hormonal das gemas durante a dormência invernal, nos deparamos com a dificuldade de extração dos metabólitos dessas amostras. As gemas não respondiam como esperado, mesmo com a utilização de diferentes protocolos amplamente utilizados. As tentativas de adaptação de outros métodos foram infrutíferas e surgiu então a necessidade de desenvolvimento de um método novo, usando como base a literatura e testando todas as informações/avanços citados.

### 5.1 MÉTODO PARA EXTRAÇÃO, PURIFICAÇÃO E DETECÇÃO/QUANTIFICAÇÃO DE HORMÔNIOS EM GEMAS DORMENTES.

No capítulo I deste trabalho, no desenvolvimento do método, foi demonstrado que boa parte do conhecimento metodológico não se aplica às gemas dormentes de macieiras. Nossos resultados mostraram que o principal fator limitante não é a extração e, sim, a purificação, sendo esse escolhido como principal meio de mitigação do efeito matriz (ME). A solução de extração não limita a extração de um tipo ou grupo de hormônios em específico, como afirmado na literatura (MA *et al.*, 2008; MÜLLER & MUNNÉ-BOSCH, 2011; TRUFELLI *et al.*, 2011). Uma possibilidade para esse tipo de resultado observado em outros trabalhos é de que nas extrações realizadas poderiam ter sido extraídas substâncias que diminuem a capacidade de detecção dos hormônios por espectrometria de massas (MS). Ou seja, esses outros métodos poderiam estar sofrendo ME para tais grupos hormonais. Entretanto, não houve uma investigação profunda do ME em tais métodos e, sim, a definição

de que a solução extrai preferencialmente alguns hormônios em detrimento de outros. Todos os hormônios analisados neste trabalho, e na maioria dos outros trabalhos, são hormônios que se encontram na forma neutra em pH abaixo de 4,8, com exceção de zeatina (de 3,9 a 5,6). Por conterem ácidos fórmico ou acético, as soluções são ácidas, assim como a solução de extração escolhida (metanol:água:ácido fórmico, 75:20:5, v:v:v), que tem pH 2. Baseado no pH, não há diferenças de extração entre os analitos. Dos métodos de extração hormonal citados anteriormente, nem todos utilizam algum método de purificação. A soma destas duas informações anteriores abre espaço para a possibilidade de que o ME tenha causado essa aparente preferência da solução de extração por alguns analitos.

A purificação dos extratos das amostras teve o maior impacto na detecção dos analitos, pois neste momento foi possível observar as maiores diferenças de recuperação hormonal entre as mudanças testadas. Há diversos métodos de purificação, sendo SPE o mais utilizado e escolhido para esse trabalho, mais especificamente com as colunas da família Oasis (MAX, HLB e MCX, Waters Corp.). Boa parte dos métodos utiliza colunas de troca iônica e utilizam as colunas de modo muito semelhante ao recomendado pelas fabricantes (Waters Corp., Sigma-Aldrich e Agilent Tech.). Entretanto, alguns detalhes são importantes de serem destacados: a preparação da coluna, o fluxo utilizado e o modo de eluição. A maioria dos métodos define a preparação das colunas como 'Umidificação' por metanol e 'Ativação' por 1 M de ácido fórmico ou acético. Como dito antes, os hormônios são em sua maioria ácidos, e ao serem carregados numa coluna acidificada acabam não se fixando e sendo eluídos como descarte, principalmente quando eles são ressuspensos em solução 1 M de ácido fórmico após a secagem do teor alcoólico. A ativação da coluna e a ressuspensão das amostras por água apresentou-se como o melhor meio para a preparação prévia destas amostras antes da eluição nas colunas de purificação. Outro ponto é a escolha da coluna que, por lógica, deveria ser uma trocadora aniônica (Oasis MAX) para analitos ácidos.

Entretanto, os resultados com amostras expõem a coluna trocadora catiônica (Oasis MCX) como a melhor opção, o que indica que há mais do que um efeito bioquímico a ser considerado no momento de escolher o meio de purificar as amostras. Após a definição da coluna e de como prepará-la, o fluxo

de eluição foi um importante fator impeditivo, já que fluxos acima da capacidade da coluna podem secá-la e, assim, acabar com a capacidade de purificação da coluna. Os diversos métodos já publicados (DOBREV *et al.*, 2005; MATSUDA *et al.*, 2005; PAN *et al.*, 2008; MÜLLER & MUNNÉ-BOSCH, 2011) não salientam a velocidade a ser utilizada, e outros sugerem altas velocidades (SUPELCO, 1998; AGILENT Corp., 2009), inclusive acima dos limites indicados pelos fabricantes. A utilização errada da coluna levou a um atraso no desenvolvimento do método, pois foi empregado a velocidade de 5 mL.min<sup>-1</sup>, recomendada por SUPELCO (1998) e por DOBREV & KAMÍNEK (2002). Após contato com a fabricante (Waters Corp.), verificou-se que essa velocidade era prejudicial para as propriedades físico-químicas da coluna. Nesta etapa de velocidade, entra também o fator “centrifugação da amostra antes de se colocar na coluna”, pois a presença de material particulado (proveniente da extração) entope a coluna, principalmente quando era utilizado ácido acético na etapa de lavagem do SPE. Diante deste ponto, após a ressuspensão em água das amostras, uma centrifugação pode ser necessária para se evitar problemas. Com a correta centrifugação e utilização de ácido fórmico na extração, não houve mais entupimento da coluna na purificação e não houve mais necessidade de utilização da bomba de vácuo, sendo a velocidade controlada apenas pela força da gravidade. Tal velocidade está dentro dos limites definidos pelo fabricante, de 2 mL.min<sup>-1</sup>. Após esse passo, a eluição foi o ponto de ajuste, já que os analitos estavam presos na coluna, mas não eram eluídos ou quando eram apresentavam muitos interferentes, como pigmentos.

Após diversos testes, verificou-se que a melhor estratégia era com eluições seriadas, que permitiam a eluição dos hormônios, mas com menor presença dos pigmentos. A Eluição 1 permaneceu sendo efetuada com metanol puro, porém foi adicionado 1 mL de água entre a Eluição 1 e a Eluição 2, sendo essa última a que mais sofreu mudanças. Foi possível diminuir a concentração de hidróxido de amônio de 1,28 M para 0,0004 - 0,04 M, muito abaixo de outros relatos dessa coluna, de 0,35 M de hidróxido de amônia (CUI *et al.*, 2015). A proporção alcoólica na eluição também foi mudada para 80% na Eluição 2, dos originais 100% de metanol em (DOBREV & KAMÍNEK, 2002; DOBREV & VANKOVA, 2012). Todas essas mudanças na purificação, além de

aumentaram a recuperação e a capacidade de detecção hormonal, também protegeram o equipamento UPLC-ESI-MS/MS de interferentes e pigmentos que poderiam restringir a vida útil da coluna e do detector.

O último passo de ajustes no protocolo foi a etapa de detecção, o qual teve que sofrer mudanças em relação às recomendações da literatura. O ME tem ação principal na transição da fase líquida (proveniente da LC) para a fase gasosa (para o MS/MS) que ocorre no ESI, restringindo a ionização dos analitos-alvo. Na LC, uma rampa de eluição menos íngreme, com maior tempo, auxiliou na separação dos analitos e dos compostos que causam o ME. A escolha dos eluentes A e B da LC também tiveram grande impacto na detecção dos analitos, sendo água (A) e acetonitrila (B) os melhores eluentes e ácido fórmico a 0,1% o melhor adjuvante. Tanto na LC quanto no SPE e na extração, o ácido fórmico desempenhou melhores resultados que o ácido acético por razões ainda desconhecidas, mas provavelmente pelo fato do ácido fórmico (pKa 3,77) ser um ácido mais forte que o acético (pKa 4,76; RIPIN & EVANS, 2006). Com esses avanços, foi possível detectar com sucesso os sete hormônios de amostras dormentes, pigmentadas e/ou lignificadas.

O método para extração, purificação e detecção/quantificação desenvolvido neste trabalho foi testado em outros tecidos além das gemas dormentes de macieira. Foram escolhidas amostras que apresentam alta pigmentação, tecidos lignificados, dormentes e/ou com alta presença de carboidratos. Foram utilizados tanto tecidos que não têm registro na literatura de análise hormonal como tecidos que já foram analisados. As amostras foram: gemas dormentes de videira (*Vitis vinifera* L., cv. Pinot Noir), gemas florais e folhas de roseira (*Rosa x kordesii*), pereira (*Pyrus communis* L.), e bergamoteiro (*Citrus bergamia*). Amostras de embriões zigóticos de *Araucaria angustifolia*, embriões somáticos de *Podocarpus lambertii* Klotzsch ex Endl., assim como seiva de macieiras em estado vegetativo foram testados também. Todas as amostras apresentaram cargas hormonais que puderam ser quantificadas, com variados balanços entre as diferentes amostras. Interessantemente, os tecidos mais 'simples', como os embrionários, não necessitaram da fase de purificação por SPE para terem seus hormônios quantificados. Estes tecidos apresentavam-se sem pigmentos, moles e ativos, o que demonstra que suas cargas de interferentes eram relativamente

pequenas em relação às suas cargas hormonais. Os outros tecidos mais 'complexos' apresentaram diferentes balanços hormonais mas, no conjunto total de todas as amostras, todos os hormônios foram detectados e quantificados. As amostras que apresentaram relatos na literatura de quantificação hormonal, apresentaram balanço hormonal muito semelhante ou maior com a utilização do método desenvolvido neste trabalho. Estes dados somados sugerem que a quantidade de falsos negativos com este método é pequena, e que quando um hormônio não é quantificável, muito provavelmente ele se encontra em quantidade ínfima ou inexistente.

Os resultados obtidos com as amostras de *Podocarpus lambertii* Klotzsch ex Endl. resultaram em um artigo científico publicado na revista *Plant Science* (FI 2016: 3.437) sob o título "Glutathione and abscisic acid supplementation influences somatic embryo maturation and hormone endogenous levels during somatic embryogenesis in *Podocarpus lambertii* Klotzsch ex Endl." (doi:10.1016/j.plantsci.2016.09.012) (artigo completo em Apêndice). Com estes dados, é possível afirmar que o método é versátil e funcional em uma ampla gama de tecidos, pouco explorados pela literatura, o que abre oportunidade de estudo e compreensão de tais tecidos.

## 5.2 BALANÇO HORMONAL DE GEMAS DORMENTES DE MACIEIRA SOB INFLUÊNCIA DE DIFERENTES PORTA-ENXERTOS

'Castel' e 'Gala' são duas cultivares de maçã com requerimento de frio contrastantes para a superação da dormência das gemas no inverno (ANZANELLO *et al.*, 2014). No inverno analisado, a alta oferta de frio para o padrão da região superou o requerimento de ambas as cultivares. A avaliação do balanço hormonal das cultivares sob influência dos porta-enxertos foi similar, mesmo com taxas de brotação diferentes entre cultivares e porta-enxertos. Tendo em vista que os hormônios analisados representam boa parte dos grupos hormonais descritos como essenciais, é possível hipotetizar que (i) outro mecanismo além do hormonal regula fortemente a brotação na dormência de gemas, ou (ii) as sensibilidades das cultivares/porta-enxertos aos hormônios são contrastantes em quantidade e/ou proporção. Hormônios são já definidos como essenciais em muitos processos de desenvolvimento vegetal (DAVIES,

2010), entretanto não há um conhecimento completo do funcionamento da regulação hormonal, gênica e metabólica na dormência. Um processo tão complexo quanto a parada total de crescimento de uma árvore para enfrentar condições climáticas extremas seguida do florescimento e reprodução exige não somente uma fina regulação hormonal como também uma inter- e intrarregulação de todos os sistemas da planta, em todos os órgãos. Com isto, apesar da modulação hormonal ser dominante em diversos mecanismos vegetais, nesse caso, os hormônios parecem não ser tão decisivos no metabolismo vegetal, dando peso à hipótese (i). Quanto à hipótese (ii), existe a possibilidade de receptores hormonais estarem em diferentes quantidades, distribuição ou afinidade pelo hormônio entre as cultivares. Se não os receptores, algum(ns) passo(s) da cascata de sinalização pode(m) estar alterado(s), de modo a ter a mesma carga hormonal, mas com ativações diferentes entre as cultivares. Além da possibilidade de polimorfismos e plasticidade fenotípica. Esta alteração tem de ser parcial para este modelo funcionar, já que a literatura demonstra que mutantes totais para receptores hormonais apresentam aumento do hormônio em questão e fenótipo excessivamente alterado (RIEFLER, 2006). Como há diversas versões de receptores e de grupos transportadores para o mesmo hormônio, existe a possibilidade de o mutante ser nulo, caso seja somente nos receptores ou transportadores hormonais. Ainda existe a possibilidade de a mutação ser em algum sítio de *cis* elemento do promotor a jusante da sinalização do hormônio. Além das mutações, existe também a regulação da expressão gênica por marcações de metilação das histonas, que podem apresentar grande efeito na regulação gênica e consequente fenótipo. Deste modo, há um grande peso na possibilidade (ii), onde diversos meios podem levar à sensibilidade diferenciada aos hormônios. Outra opção plausível para essa diferença de brotação entre 'Castel' e 'Gala', mesmo com frio elevado, é de ter ocorrido o consumo de açúcares pelas gemas laterais das estacas de 'Gala', o que drena a fonte energética das gemas apicais, que foram as avaliadas. Há uma evidência que boa parte do controle apical se deve ao controle de açúcar (MASON *et al.*, 2014) e, como as estacas de 'Gala' mostraram alta brotação lateral, esse fato pode ter influenciado também a baixa brotação apical, sendo possivelmente

essa baixa brotação um artefato do experimento, não desqualificando a possibilidade (ii) como principal divergência hormonal entre 'Gala' e 'Castel'.

Os dados hormonais obtidos em um ano de frio suficiente para suprir o requerimento de frio definido demonstram que no começo da dormência não há uma grande presença hormonal e, conseqüentemente, nenhum controle fundamental. Neste ponto deve estar ocorrendo a dormência de fato, com parada quase que total do metabolismo. Como discutido no Capítulo II, provavelmente essa fase é majoritariamente controlada pelos genes *MdDAM*, cujo papel na dormência das *Rosaceae* já foi bem demonstrado (WISNIEWSKI *et al.*, 2011; WISNIEWSKI *et al.*, 2015; PORTO *et al.*, 2016; TUAN *et al.*, 2017).

É a partir do fim da endodormência que se passa a ter a presença hormonal em maior quantidade. ABA assume o papel principal em 496 CH nas combinações 'Castel'/M9, 'Castel'/MaM9, 'Gala'/M9 e 'Gala'/Maruba. Esta presença de ABA ocorre exatamente no ponto anterior à brotação nas plantas 'Castel'. 'Castel'/Maruba, que não apresentou esse pico de ABA em 496 CH, passou para ecodormência neste ponto, antes das outras combinações de 'Castel'. Nessa fase, ocorre também a presença de GA<sub>4</sub> e Z. GA<sub>4</sub> possivelmente está envolvido no desmantelamento da barreira de calose (RINNE *et al.*, 2011) e, conseqüentemente, na importação de Z de fora da gema. Com a indicação de que os genes *DAM* induzem ABA e ABA reprime *DAM* em pera (TUAN *et al.*, 2017), e de que *DAM* tem *cis* elementos para ARR-tipo B, que são parte da rota de sinalização de Z, é possível que nessa fase tanto ABA quanto Z estejam reprimindo *DAM*, porém ABA estaria reprimindo a brotação antecipada. Fica visível que ABA, neste ponto em diante, é proveniente das raízes, uma vez que os porta-enxertos mantêm padrões muito semelhantes entre as cultivares. Com isso é possível definir o impacto dos porta-enxertos na brotação, uma vez que M9 atrasou a brotação, enquanto que Maruba parece não atrasar, mas sim manter o padrão natural para a gema. A partir deste momento, ocorre o processo já descrito para o metabolismo de superação da dormência em sementes: diminuição da presença de ABA e aumento da presença de GA, culminando na germinação (DEBSKA *et al.*, 2013; WANG *et al.*, 2016), no modelo 'gangorra de ABA-GA' (Figura 2).

É importante salientar que essa modulação parece atuar como uma resposta de ativação do metabolismo de forma independente ao frio,

reprimindo-o para não brotar antecipadamente e induzindo a brotação na época certa, porém não parece controlar a endodormência e, sim, a ecodormência. Dito isto, espera-se que a endodormência seria majoritariamente regulada por fatores gênicos e possivelmente por carboidratos, enquanto a ecodormência por fatores metabólicos, majoritariamente hormonal. Considerando essa hipótese como verdadeira, a diferença da necessidade de frio entre 'Castel' e 'Gala' poderia ser resumida ao controle gênico da endodormência, mais antecipada em 'Castel', uma vez que ambas apresentaram metabolismos hormonais muito semelhantes, porém com fenologia contrastante. Não sendo as diferenças entre as cultivares no controle gênico da endodormência, aumenta a possibilidade de estar correta a possibilidade (ii) acima citada, da sensibilidade diferenciada aos metabólitos.

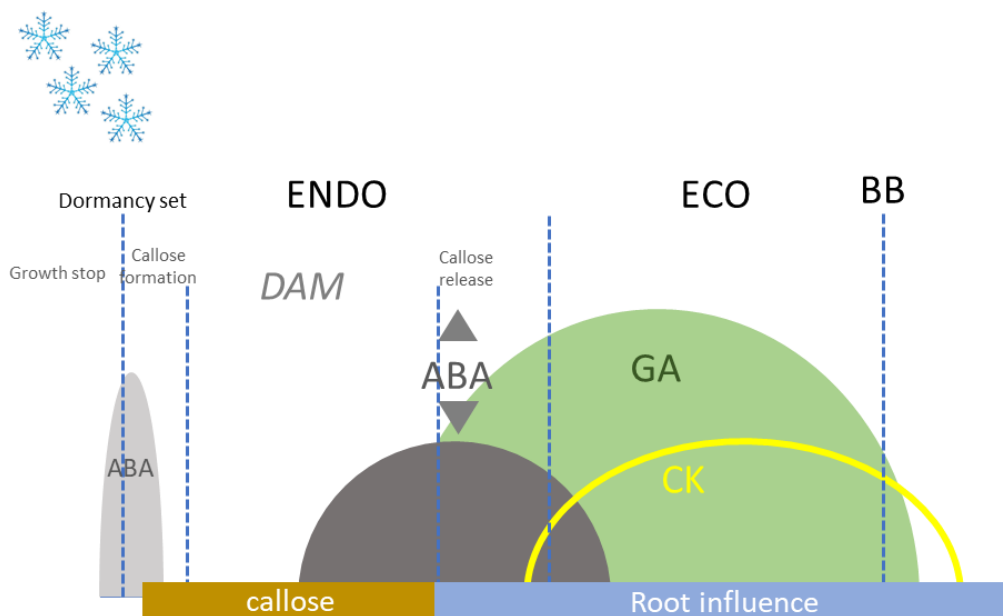


Figura 2: Modelo de balanço hormonal no contexto da dormência com alta oferta de frio. A entrada da dormência é caracterizada pela presença de ABA em altas quantidades e formação da barreira de calose. Em seguida não há registro de presença significativa de hormônios, mas já foi reportado a presença de *DAM* na endodormência. No fim da endodormência, há o dismantelamento da barreira de calose e entrada de ABA proveniente das raízes. A quantidade de ABA é variável de acordo com o porta-enxerto (indicado pelas setas). A partir da transição de endo- para ecodormente, há a presença de giberelinas e citocininas também, que são parte do desenvolvimento da ecodormência. A influência das raízes (e porte-enxertos) se dá na ecodormência, podendo atrasar brotação com aporte de ABA.

Outro ponto de importância que não pode ser ignorado, é o quanto o metabolismo está ativo nas duas cultivares. 'Castel' tem uma dormência com



maior atividade metabólica, considerando-se um trabalho anterior que demonstra que a quantidade de glutathione reduzida e oxidada é maior em 'Castel' do que em 'Gala'. 'Castel' também brotou com uma quantidade menor de glutathione reduzida e oxidada que 'Gala', o que demonstra um meristema ativo e mais sensível aos metabólitos (SANTOS *et al.*, 2011). Com isto, a diferença entre as taxas de brotações entre as cultivares deve-se também a mecanismos outros que não os hormonais. A visualização da influência do porta-enxerto na fenologia da gema torna-se então melhor visualizada isolando-se a cultivar 'Castel', que apresenta reação hormonal e fisiológica aos porta-enxertos.

A dormência em macieira é majoritariamente estudada nas gemas, porém a planta como um todo entra em dormência. Portanto, um ponto importante a ser considerado é o porta-enxerto utilizado. Porta-enxertos são escolhidos por seu vigor de crescimento, sendo a preferência por variedades ananizantes para facilitar o manuseio da copa e a colheita. Assim como as cultivares, os porta-enxertos também apresentam requerimento de frio para quebrar a dormência, porém ainda não se sabe como isso é controlado nas raízes e sua relação com as gemas. O que foi observado neste trabalho é que Maruba induziu uma brotação da copa 'Castel' mais antecipada em inverno com alta disponibilidade de frio em relação aos outros porta-enxertos. Maruba também não apresentou o pico de ABA em 496 CH, quando atinge a ecodormência, o que mostra que nessa fase ABA é de fato o repressor majoritário da brotação. O porta-enxerto M9, que é ananizante supostamente por restrições do sistema vascular (HOOIJDONK *et al.*, 2011; TWORKOSKI & FAZIO, 2011), foi o último a atingir a ecodormência e apresentou taxas de brotação mais baixas que os outros. M9 também apresentou a maior presença de ABA em 496 CH, o que indica mais uma vez que ABA é o controlador principal neste ponto. M9 já foi relatado como o porta-enxerto com maior quantidade de ABA na seiva bruta, numa comparação com o porta-enxerto M111, que é vigoroso (TWORKOSKI & FAZIO, 2011, 2016). MaM9 apresenta o balanço hormonal semelhante a Maruba, porém com fenologia próxima de M9, apesar de ter maior quantidade de GA<sub>4</sub> em 707 CH. A diferença entre o comportamento das cultivares é influenciada metabolicamente na regulação da quantidade de ABA, que se reflete fenologicamente no atraso ou antecipação

da brotação. Interessantemente, com o isolamento da gema na dormência pela barreira de calose, não deveria haver influência do porta-enxerto na copa, porém a mudança se dá exatamente no ponto de 496 CH, quando possivelmente a barreira da calose é desmantelada por  $\beta$ -1,3-glucanases, que são induzidas por GAs. Supõe-se, então, que a endodormência ocorre de fato de forma isolada por calose, mas a ecodormência recebe significativa influência da raiz. Este bloqueio da brotação da gema pela raiz via hormonal demonstra que a quebra de dormência se dá como um todo, e que as raízes desempenham papel importante nesse mecanismo. No estado normal, as raízes devem agir como sinalizadoras para a parte aérea de que é possível o crescimento sustentável, ou bloqueando o crescimento com o uso hormonal, como citocininas, giberelinas e ácido abscísico (BJO *et al.*, 2007; WESTON *et al.*, 2009; NASEEM & DANDEKAR, 2012; KIEBER & SCHALLER, 2014). Esse sistema pode ser expandido para o estado da dormência, onde as raízes podem agir como repressoras ou estimuladoras de crescimento do ápice e, portanto, também devem apresentar um sistema de percepção de frio e à disponibilidade de água no solo.

A dormência da macieira como um todo apresenta um intrincado e complexo sistema, que não pode ser explicado unicamente por uma via metabólica, gênica ou proteica. Esse sistema está mais claro com os novos dados de balanço hormonal e as influências que este recebe de agentes externos como a alta oferta de frio, os porta-enxertos e o empilhamento de reações fisiológicas às mudanças ambientais. Porém há muito a ser estudado para a compreensão refinada da modulação hormonal na dormência da macieira e da família das *Rosacea*, para ser desenvolvida uma ferramenta biotecnológica de aprimoramento dessas culturas e, conseqüentemente, dos ajustes ao novo clima que está se formando com o aquecimento global.

## 6 PERSPECTIVAS

- Desenvolver o protocolo de detecção/quantificação para outras formas hormonais dentro dos mesmos grupos já identificados;
- Identificar e quantificar as formas de conjugados hormonais, que podem estar presentes durante o período hormonal;
- Análise do balanço hormonal na dormência de gemas de macieira com menor intervalo de tempo entre os pontos de coleta;
- Analisar o balanço hormonal de outras macieiras e pereiras, com requerimentos de frio diferentes e em condições de invernos de alta e baixa oferta de frio;
- Avaliar os promotores e genes das rotas metabólicas dos hormônios essenciais da dormência e comparar entre as cultivares e porta-enxertos.
- Avaliar a presença de calose na dormência e sua resposta aos hormônios em invernos com alta e baixa oferta de frio;
- Avaliar modulação hormonal nas gemas e seiva dos porta-enxertos durante a dormência hiberna e fase de crescimento ativo

## 7 REFERÊNCIAS BIBLIOGRÁFICAS

ALVAREZ, R.; NISSEN, S. J.; SUTTER, E. G. Relationship between Indole-3-Acetic Acid Levels in Apple (*Malus pumila* Mill) Rootstocks Cultured in Vitro and Adventitious Root Formation in the Presence of Indole-3-Butyric Acid. *Plant physiology*, 89(2): 439–443, 1989.

ANZANELLO, R.; FIALHO, F. B.; DOS SANTOS, H. P.; BERGAMASCHI, H.; MARODIN, G. A. B. Bud dormancy in apple trees after thermal fluctuations. *Pesquisa Agropecuaria Brasileira*, 49(6): 457–464, 2014.

AOAC INTERNATIONAL METHODS COMMITTEE GUIDELINES FOR VALIDATION OF QUALITATIVE AND QUANTITATIVE FOOD MICROBIOLOGICAL OFFICIAL METHODS OF ANALYSIS. Guidelines for Single Laboratory Validation (SLV) of Chemical Methods for Metals in Food, 2002.

ARORA, R.; ROWLAND, L. J.; TANINO, K. Induction and release of bud dormancy in woody perennials: A science comes of age. *HortScience*, 38(5): 911–921, 2003.

BAI, S.; SAITO, T.; SAKAMOTO, D.; ITO, A.; FUJII, H.; MORIGUCHI, T. Transcriptome analysis of japanese pear (*Pyrus pyrifolia* Nakai) flower buds transitioning through endodormancy. *Plant and Cell Physiology*, 54(7): 1132–1151, 2013.

BJÖRKLUND, S.; ANTTI, H.; UDDESTRAND, I.; MORITZ, T.; SUNDBERG, B. Cross-talk between gibberellin and auxin in development of *Populus* wood: gibberellin stimulates polar auxin transport and has a common transcriptome with auxin. *The Plant Journal*, 52(3): 499–511, 2007.

BOSCO, R.; DAESELEIRE, E.; VAN PAMEL, E.; SCARIOT, V.; LEUS, L. Development of an ultrahigh-performance liquid chromatography-electrospray ionization-tandem mass spectrometry method for the simultaneous determination of salicylic acid, jasmonic acid, and abscisic acid in rose leaves. *Journal of Agricultural and Food Chemistry*, 62(27): 6278–6284, 2014.

BYLDA, C.; THIELE, R.; KOBOLD, U.; VOLMER, D. A. Recent advances in sample preparation techniques to overcome difficulties encountered during quantitative analysis of small molecules from biofluids using LC-MS/MS. *The Analyst*, 139(10): 2265, 2014.

CHAMBERS, E.; WAGROWSKI-DIEHL, D. M.; LU, Z.; MAZZEO, J. R. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *Journal of Chromatography B*, 852: 22–34, 2007.

CHANDLER, W. H. Chilling requirements for opening of buds on deciduous orchard trees and some other plants in California. *Bulletin 611*, 611:3–11, 1937.

CORNILLE, A.; GIRAUD, T.; SMULDERS, M. J. M.; ROLDÁN-RUIZ, I.; GLADIEUX, P. The domestication and evolutionary ecology of apples. *Trends in Genetics*, 30(2): 57–65, 2014.

CUI, K.; LIN, Y.; ZHOU, X.; LI, S.; LIU, H.; ZENG, F.; ZHU, F.; OUYANG, G.; ZENG, Z. Comparison of sample pretreatment methods for the determination of multiple phytohormones in plant samples by liquid chromatography-electrospray ionization-tandem mass spectrometry. *Microchemical Journal*, 121: 25–31, 2015.

DAVIES, P. J. The Plant Hormones: Their Nature, Occurrence, and Functions. In: *Plant Hormones*. Dordrecht: Springer Netherlands, p. 1–15, 2010.

DEBSKA, K.; KRASUSKA, U.; BUDNICKA, K.; BOGATEK, R.; GNIAZDOWSKA, A. Dormancy removal of apple seeds by cold stratification is associated with fluctuation in H<sub>2</sub>O<sub>2</sub>, NO production and protein carbonylation level. *Journal of Plant Physiology*, 170(5): 480–488, 2013.

DOBREV, P. I.; VANKOVA, R. Quantification of Abscisic Acid, Cytokinin, and Auxin Content in Salt-Stressed Plant Tissues. In: SHABALA, S.; CUIN, T. A. (Ed.). *Plant Salt Tolerance: Methods and Protocols*. Totowa, Humana Press, p. 251–261, 2012a.

DUN, E. A.; BREWER, P. B.; BEVERIDGE, C. A. Strigolactones: discovery of the elusive shoot branching hormone. *Trends in Plant Science*, 14(7):364-372, 2009.

FALAVIGNA, V. da S.; PORTO, D. D.; BUFFON, V.; MARGIS-PINHEIRO, M.; PASQUALI, G.; REVERS, L. F. Differential Transcriptional Profiles of Dormancy-Related Genes in Apple Buds. *Plant Molecular Biology Reporter*, 32(4): 796–813, 2014.

FALAVIGNA, V. S.; PORTO, D. D.; SILVEIRA, C. P.; REVERS, L. F. Recent advances in genetics and molecular control of bud dormancy in pipfruits. *Advances in Plant Dormancy*, 1: 107–122, 2015.

FIORAVANÇO, J. C.; CZERMAINSKI, A. B. C.; OLIVEIRA, P. R. D. de. Yield efficiency for nine apple cultivars grafted on two rootstocks. *Ciência Rural*, 46(10): 1701–1706, 2016.

GILAR, M.; JAWORSKI, A.; MCDONALD, T. S. Solvent selectivity and strength in reversed-phase liquid chromatography separation of peptides. *Journal of Chromatography A*, 1337: 140–146, 2014.

GUSTAVSSON, S. Å.; SAMSKOG, J.; MARKIDES, K. E.; LÅNGSTRÖM, B. Studies of signal suppression in liquid chromatography–electrospray ionization mass spectrometry using volatile ion-pairing reagents. *Journal of Chromatography A*, 937(1–2): 41–47, 2001.

HAN, Z.; LIU, G.; RAO, Q.; BAI, B.; ZHAO, Z.; LIU, H.; WU, A. A liquid chromatography tandem mass spectrometry method for simultaneous determination of acid/alkaline phytohormones in grapes. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 881–882: 83–89, 2012.

HOOIJDONK, B. Van; WOOLLEY, D.; WARRINGTON, I.; TUSTIN, S. Rootstocks Modify Scion Architecture, Endogenous Hormones, and Root Growth of Newly Grafted “Royal Gala” Apple Trees. *Journal of American Horticultural Science*, 136(2): 93–102, 2011.

HOU, R.; JIAO, W.; XIAO, Y.; GUO, J.; LV, Y.; TAN, H.; HU, J.; WAN, X. Novel use of PVPP in a modified QuEChERS extraction method for UPLC-MS/MS analysis of neonicotinoid insecticides in tea matrices. *Analytical Methods*, 7(13): 5521–5529, 2015.

IVANOV DOBREV, P.; KAMÍNEK, M. Fast and efficient separation of cytokinins from auxin and abscisic acid and their purification using mixed-mode solid-phase extraction. *Journal of Chromatography A*, 950(1–2): 21–29, 2002.

KIEBER, J. J.; SCHALLER, G. E. Cytokinins. *The Arabidopsis Book*, 12: e0168, 2014.

KUMAR, G.; GUPTA, K.; PATHANIA, S.; SWARNKAR, M. K.; RATTAN, U. K.; SINGH, G.; SHARMA, R. K.; SINGH, A. K. Chilling Affects Phytohormone and Post-Embryonic Development Pathways during Bud Break and Fruit Set in Apple (*Malus domestica* Borkh.). *Scientific Reports*, 7: 42593, 2017.

LABUSCHAGNÉ, I. F.; LOUW, J. H.; SCHMIDT, K.; SADIE, A. Genetic Variation in Chilling Requirement in Apple Progeny. *Journal of the American Society for Horticultural Science*, 127(4): 663–672, 2002.

LAMBERT, W. E.; MURPHY, C. M Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. *Journal of the American Society for Mass Spectrometry*. 14: 1290–1294, 2003.

LANG, G.; EARLY, J.; MARTIN, G.; DARNELL, R. Endo-, para-, and ecodormancy: physiological terminology and classification for dormancy research. *HortScience*, 22(3): 371–377, 1987.

LEIDA, C.; TEROL, J.; MARTÍ, G.; AGUSTÍ, M.; LLÁCER, G.; BADENES, M. L.; RÍOS, G. Identification of genes associated with bud dormancy release in *Prunus persica* by suppression subtractive hybridization. *Tree Physiology*, 30(5): 655–666, 2010.

LJUNG, K.; SANDBERG, G.; MORITZ, T. Methods of Plant Hormone Analysis. In: *Plant Hormones*. Dordrecht, Springer Netherlands. p. 717–740, 2010

MA, Z.; GE, L.; LEE, A. S. Y.; YONG, J. W. H.; TAN, S. N.; ONG, E. S. Simultaneous analysis of different classes of phytohormones in coconut (*Cocos nucifera* L.) water using high-performance liquid chromatography and liquid chromatography-tandem mass spectrometry after solid-phase extraction. *Analytica Chimica Acta*, 610(2): 274–281, 2008.

MASON, M. G.; ROSS, J. J.; BABST, B. A.; WIENCLAW, B. N.; BEVERIDGE, C. A. Sugar demand, not auxin, is the initial regulator of apical dominance. *Proceedings of the National Academy of Sciences*, 111(16): 6092–6097, 2014.

MATUSZEWSKI, B. K.; CONSTANZER, M. L. Matrix Effect in Quantitative LC / MS / MS Analyses of Biological Fluids : A Method for Determination of Finasteride in Human Plasma at Picogram Per Milliliter Concentrations. *Analytical Chemistry*. 2700(10): 1001–1008, 1998.

MATUSZEWSKI, B. K.; CONSTANZER, M. L.; CHAVEZ-ENG, C. M. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry*, 75(13): 3019–3030, 2003.

MÜLLER, M.; MUNNÉ-BOSCH, S. Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry. *Plant methods*, 7: 37, 2011.

NASEEM, M.; DANDEKAR, T. The Role of Auxin-Cytokinin Antagonism in Plant-Pathogen Interactions. *PLoS Pathogens*, 8(11): e1003026, 2012.

NIU, Q.; ZONG, Y.; QIAN, M.; YANG, F.; TENG, Y. Simultaneous quantitative determination of major plant hormones in pear flowers and fruit by UPLC/ESI-MS/MS. *Analytical Methods*, 6(6): 1766, 2014.

PORTO, D. D.; BRUNEAU, M.; PERINI, P.; ANZANELLO, R.; RENOU, J. P.; DOS SANTOS, H. P.; FIALHO, F. B.; REVERS, L. F. Transcription profiling of the chilling

requirement for bud break in apples: A putative role for FLC-like genes. *Journal of Experimental Botany*, 66(9): 2659–2672, 2015.

PORTO, D. D.; FALAVIGNA, V. da S.; ARENHART, R. A.; PERINI, P.; BUFFON, V.; ANZANELLO, R.; DOS SANTOS, H. P.; FIALHO, F. B.; DE OLIVEIRA, P. R. D.; REVERS, L. F. Structural genomics and transcriptional characterization of the Dormancy-Associated MADS-box genes during bud dormancy progression in apple. *Tree Genetics and Genomes*, 12(3): 15, 2016.

RIEFLER, M. Arabidopsis Cytokinin Receptor Mutants Reveal Functions in Shoot Growth, Leaf Senescence, Seed Size, Germination, Root Development, and Cytokinin Metabolism. *the Plant Cell Online*, 18(1): 40–54, 2006.

RINNE, P. L. H.; WELLING, A.; VAHALA, J.; RIPEL, L.; RUONALA, R.; KANGASJÄRVI, J.; VAN DER SCHOOT, C. Chilling of Dormant Buds Hyperinduces FLOWERING LOCUS T and Recruits GA-Inducible 1,3- $\beta$ -Glucanases to Reopen Signal Conduits and Release Dormancy in *Populus*. *The Plant Cell*, 23(1): 130–146, 2011.

RIPIN, D. H.; EVANS, D. A. PKa table - Chemistry 206. *Advanced Organic Chemistry*, 206: 855–881, 2006.

ROHDE, A.; BHALERAO, R. P. Plant dormancy in the perennial context. *Trends in Plant Science*, 12(5): 217–223, 2007.

ROSS, J. J.; WESTON, D. E.; DAVIDSON, S. E.; REID, J. B. Plant hormone interactions: How complex are they? *Physiologia Plantarum*, 141(4):299-309, 2011. .

RUTTINK, T.; AREND, M.; MORREEL, K.; STORME, V.; ROMBAUTS, S.; FROMM, J.; BHALERAO, R. P.; BOERJAN, W.; ROHDE, A. A Molecular Timetable for Apical Bud Formation and Dormancy Induction in Poplar. *the Plant Cell Online*, 19(8): 2370–2390, 2007.

SANTOS, H. P.; REVERS, L. F.; LEITE, G. B.; BERNARDI, J. Caracterização da dormência hiberna em gemas de macieira. In: NACHTIGALL, G. R. (Ed.). *Inovações tecnológicas para o setor da maçã - Inovamaçã*. Cap.9: 257–273. 2007

SINGH, R. K.; SVYSTUN, T.; ALDAHMAH, B.; JÖNSSON, A. M.; BHALERAO, R. P. Photoperiod- and temperature-mediated control of phenology in trees – a molecular perspective. *New Phytologist*, 213(2): 511–524, 2017.

SUPELCO; Bulletin 910. *Guide to Solid Phase Extraction*, Sigma-Aldrich Co. 1998.

SZECSKÓ, V.; HROTKÓ, K.; STEFANOVITS-BÁNYAI, E. Phenolic Compounds, Bud Dormancy, and Rooting Ability of Plum Hardwood Cuttings. *Acta Horticulturae*, 658: 679–687, 2004.

TRUFELLI, H.; PALMA, P.; FAMIGLINI, G.; CAPPIELLO, A. An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrometry Reviews*, 30(3): 491–509, 2011.

TUAN, P. A.; BAI, S.; SAITO, T.; ITO, A.; MORIGUCHI, T. Dormancy-Associated MADS-Box (DAM) and the Abscisic Acid Pathway Regulate Pear Endodormancy Through a Feedback Mechanism. *Plant and Cell Physiology*, 0(0): 1–13. 2017.

TWORKOSKI, T.; FAZIO, G. Physiological and morphological effects of size-controlling rootstocks on “Fuji” apple scions. *Acta Horticulturae*, 903(903): 865–872, 2011.

TWORKOSKI, T.; FAZIO, G. Effects of Size-Controlling Apple Rootstocks on Growth, Abscisic Acid, and Hydraulic Conductivity of Scion of Different Vigor. *International Journal of Fruit Science*, 15(4): 369–381, 2015.

TWORKOSKI, T.; FAZIO, G. Hormone and growth interactions of scions and size-controlling rootstocks of young apple trees. *Plant Growth Regulation*, 78(1): 105–119, 2016.

VATS, P.; VERMA, S. M.; MONIF, T. Mechanistic Evaluation of Matrix Effect on Three Different Model of Mass Spectrometer by Using a Model Drug. *Journal of Analytical & Bioanalytical Techniques* 7(3):11, 2016.

WANG, D.; GAO, Z.; DU, P.; XIAO, W.; TAN, Q.; CHEN, X.; LI, L.; GAO, D. Expression of ABA Metabolism-Related Genes Suggests Similarities and Differences Between Seed Dormancy and Bud Dormancy of Peach (*Prunus persica*). *Frontiers in Plant Science*, 6: 1–17, 2016.

WESTON, D. E.; REID, J. B.; ROSS, J. J. Auxin regulation of gibberellin biosynthesis in the roots of pea (*Pisum sativum*). *Functional Plant Biology*, 36(4): 362, 2009.

WILKINSON, S.; DAVIES, W. J. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. *Plant, Cell and Environment*, 33(4): 510–525, 2010.

WISNIEWSKI, M.; NORELLI, J.; ARTLIP, T. Overexpression of a peach CBF gene in apple: a model for understanding the integration of growth, dormancy, and cold hardiness in woody plants. *Plant Physiology*, 6: 85, 2015.

WISNIEWSKI, M.; NORELLI, J.; BASSETT, C.; ARTLIP, T.; MACARISIN, D. Ectopic expression of a novel peach (*Prunus persica*) CBF transcription factor in apple (*Malus x domestica*) results in short-day induced dormancy and increased cold hardiness. *Planta*, 233(5): 971–983, 2011.

ZHOU, R.; SQUIRES, T. M.; AMBROSE, S. J.; ABRAMS, S. R.; ROSS, A. R. S.; CUTLER, A. J. Rapid extraction of abscisic acid and its metabolites for liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 1010(1): 75–85, 2003.



## CURRICULUM VITÆ resumido

GARIGHAN, J.

### 1. DADOS PESSOAIS

**Nome:** Julio de Andrade Garighan

**Local e data de nascimento:** Jaguarão, RS, Brasil. 05/04/1992

**E-mail:** julio.garighan@outlook.com

### 2. FORMAÇÃO:

2015-2017

**Mestrado em Biologia Celular e Molecular**, PPGBCM, Universidade Federal do Rio Grande do Sul, UFRGS, Brasil. Caracterização do Balanço Hormonal de Gemas durante o Processo de Dormência em Macieira. Orientadores: Dr. Giancarlo Pasquali, Dr. Luis Fernando Revers e Dr Henrique Pessoa dos Santos.

2010-2014

**Bacharel em Biotecnologia Molecular**, Universidade Federal do Rio Grande do Sul, UFRGS, Brasil. Análise da região promotora do gene OsAPX3 que codifica uma ascorbato peroxidase peroxissomal em arroz. Orientadora: Dra. Márcia Pinheiro-Margis.

### 3. ESTÁGIOS:

2013 – 2014

Estágio de iniciação científica no Núcleo de Genômica Funcional de Plantas, UFRGS, Brasil.

Orientação: Dra. Marcia Maria Auxiliadora Naschenveng Pinheiro Margis e Dra. Gisele Passaia.

Bolsista PIBIC CNPq – UFRGS

Análise dos promotores dos genes Ascorbato Peroxidase 1, 3 e 4 de arroz. Pesquisa integrante do projeto “Interação entre estresse oxidativo e estresse abiótico em arroz (*Oryza sativa*)”.

2013 – 2013

Estágio de iniciação científica no Núcleo de Genômica Funcional de Plantas, UFRGS, Brasil.

Orientação: Dra. Marcia Maria Auxiliadora Naschenveng Pinheiro Margis, Dra. Gisele Passaia e Dr Rafael Augusto Arenhart.

Bolsista PIBIC CNPq – UFRGS

Pesquisa integrante do projeto “Caracterização funcional dos genes ASR na resposta ao alumínio em arroz (*Oryza sativa*)”.

2011 – 2012

Estágio de iniciação científica no Núcleo de Genômica Funcional de Plantas, UFRGS, Brasil.

Orientação: Dra. Marcia Maria Auxiliadora Naschenveng Pinheiro Margis e Dra. Carolina Werner Ribeiro.

Bolsista PROBIC FAPERGS - UFRGS

Análise dos promotores dos genes Ascorbato Peroxidase 1, 3 e 4 de arroz. Pesquisa integrante do projeto “Interação entre estresse oxidativo e estresse abiótico em arroz (*Oryza sativa*)”.

2010 - 2011

Estágio de iniciação científica no Núcleo de Genômica Funcional de Plantas, UFRGS, Brasil.

Orientação: Dra. Marcia Maria Auxiliadora Naschenveng Pinheiro Margis e Dra. Carolina Werner Ribeiro.

Bolsista PROBIC FAPERGS - UFRGS

Análise dos promotores dos genes Ascorbato Peroxidase 1, 3 e 4 de arroz. Pesquisa integrante do projeto “Interação entre estresse oxidativo e estresse abiótico em arroz (*Oryza sativa*)”.

#### 4. PRÊMIOS E DISTINÇÕES

2013

Trabalho Destaque na seção ‘Genética Vegetal’ do Salão de Iniciação Científica da UFRGS.

#### 5. ARTIGOS COMPLETOS PUBLICADOS

RIBEIRO, C. W.; KORBES, A. P.; GARIGHAN, J. A.; JARDIM-MESSEDER, D.; CARVALHO, F. E. L.; SOUSA, R. H. V.; CAVERZAN, A.; TEIXEIRA, F. K.; SILVEIRA, J. A. G.; MARGIS-PINHEIRO, M. Rice peroxisomal ascorbate peroxidase knockdown affects ROS signaling and triggers early leaf senescence. *Plant Science*, 263: 55–65, 2017.

- [1] V. Szecskó, K. Hrotkó, E. Stefanovits-Bányai, PHENOLIC COMPOUNDS, BUD DORMANCY, AND ROOTING ABILITY OF PLUM HARDWOOD CUTTINGS, *Acta Hort.* (2004) 679–687. doi:10.17660/ActaHortic.2004.658.104.
- [2] M. Naseem, T. Dandekar, The Role of Auxin-Cytokinin Antagonism in Plant-Pathogen Interactions, *PLoS Pathog.* 8 (2012) e1003026. doi:10.1371/journal.ppat.1003026.
- [3] J.J. Kieber, G.E. Schaller, Cytokinins, *Arab. B.* 12 (2014) e0168. doi:10.1199/tab.0168.
- [4] D.E. Weston, J.B. Reid, J.J. Ross, Auxin regulation of gibberellin biosynthesis in the roots of pea (*Pisum sativum*), *Funct. Plant Biol.* 36 (2009) 362–369. doi:10.1071/FP08301.
- [5] S. Björklund, H. Antti, I. Uddestrand, T. Moritz, B. Sundberg, Cross-talk

between gibberellin and auxin in development of *Populus* wood: gibberellin stimulates polar auxin transport and has a common transcriptome with auxin, *Plant J.* 52 (2007) 499–511. doi:10.1111/j.1365-313X.2007.03250.x.

## 6. RESUMOS E TRABALHOS APRESENTADOS EM CONGRESSOS

GARIGHAN, J.; RIBEIRO, C. W.; MARGIS-PINHEIRO, M. Estudo de regiões promotoras das isoformas citosólicas e peroxissomais de ascorbato peroxidase de arroz (*Oryza sativa* L.). *In: XXIII Salão de Iniciação Científica da UFRGS.* Porto Alegre. 2011.

GARIGHAN, J.; RIBEIRO, C. W.; MARGIS-PINHEIRO, M. Estudo de Regiões Promotoras dos Genes das Isoformas Citosólica e Peroxissomais de Ascorbato Peroxidase de Arroz (*Oryza sativa* L.). *In: XXIV Salão de Iniciação Científica da UFRGS.* Porto Alegre. 2012.

RIBEIRO, C. W.; GARIGHAN, J.; ROSA, S. B.; SILVEIRA, JOAQUIM A. G.; SILVERIO, A.; MARIATH, J.; ALVES-FERREIRA, M.; MARGIS-PINHEIRO, M. Functional analyses of peroxisomal ascorbate peroxidase 4 in rice (*Oryza sativa* L.). *In: 58º Congresso Brasileiro de Genética.* Foz do Iguaçu. 2012.

RIBEIRO, C. W.; GARIGHAN, J.; ROSA, S. B.; SILVEIRA, JOAQUIM A. G.; SILVERIO, A.; MARIATH, J.; ALVES-FERREIRA, M.; MARGIS-PINHEIRO, M. Functional characterization of the rice peroxisomal ascorbate peroxidase 4. *In: IV Simpósio Brasileiro de Genética Molecular de Plantas.* Bento Gonçalves. 2013.

GARIGHAN, J.; RIBEIRO, C. W.; MARGIS-PINHEIRO, M. Estudo da região promotora da isoforma citosólica de ascorbato peroxidase de arroz (*Oryza sativa* L.). *In: XXV Salão de Iniciação Científica da UFRGS.* Porto Alegre. 2013.

GARIGHAN, J.; RIBEIRO, C. W.; MARGIS-PINHEIRO, M. Promoter analysis of the *OsAPx3* and *OsAPx4* genes in rice. *In: IV Simpósio Brasileiro de Genética Molecular de Plantas.* Bento Gonçalves. 2013.

GARIGHAN, J.; RIBEIRO, C. W.; MARGIS-PINHEIRO, M. Investigating the expression pattern of the *OsAPx1* gene promoter in rice. *In: 5º Congresso Brasileiro de Biotecnologia.* Florianópolis. 2013.

GARIGHAN, J.; PASSAIA, G.; MARGIS-PINHEIRO, M. Análise da região promotora do gene *OsAPx3* que codifica uma ascorbato peroxidase peroxissomal em arroz. *In: XXV Salão de Iniciação Científica da UFRGS.* Porto Alegre. 2014.

GARIGHAN, J.; PASQUALI, G.; REVERS. L.; PORTO, D.; SOUZA, D.; SANTOS, H. Hormonal balance characterization of dormant buds in apple. *In: International Plant Molecular Biology Congress. Foz do Iguaçu. 2015*

GARIGHAN, J.; SOUZA, D.; PASQUALI, G.; REVERS. L.; SANTOS, H. Estabelecimento de protocolo para análise multi-hormonal em gemas de macieira durante a dormência. *In: 13° Encontro de Iniciação Científica e 9° Encontro de Pós-Graduandos da Embrapa Uva e Vinho, Bento Gonçalves. 2015.*

GARIGHAN, J.; PASQUALI, G.; REVERS. L.; PORTO, D.; SOUZA, D.; SANTOS, H. A protocol for multihormone extraction and hormonal balance characterization of dormant buds in apple. *In: 1° Congresso de Biotecnologia da Região Sul. Lageado. 2016*

GARIGHAN, J.; PASQUALI, G.; REVERS. L.; SOUZA, D.; SANTOS, H. Estabelecimento de protocolo para análise multi-hormonal em gemas de macieira durante o período de endodormência. *In: 14° Encontro de Iniciação Científica e 10° Encontro de Pós-Graduandos da Embrapa Uva e Vinho, Bento Gonçalves. 2016.*

GARIGHAN, J.; SOUZA, D.; PASQUALI, G.; REVERS. L.; SANTOS, H. Development of a hormone extraction and quantification method on complex plant tissues. *In: XVI Encontro do PPGBCM. Porto Alegre. 2016*



## Glutathione and abscisic acid supplementation influences somatic embryo maturation and hormone endogenous levels during somatic embryogenesis in *Podocarpus lambertii* Klotzsch ex Endl.

Hugo Pacheco de Freitas Fraga<sup>a</sup>, Leila do Nascimento Vieira<sup>a</sup>, Catarina Corrêa Puttkammer<sup>a</sup>, Henrique Pessoa dos Santos<sup>b</sup>, Julio de Andrade Garighan<sup>b</sup>, Miguel Pedro Guerra<sup>a,\*</sup>

<sup>a</sup> Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Florianópolis, SC, 88034-001, Brazil

<sup>b</sup> Laboratório de Fisiologia Vegetal, Empresa Brasileira de Pesquisa Agropecuária, Empresa Uva e Vinho, Bento Gonçalves, RS, 95700-000, Brazil

### ARTICLE INFO

**Article history:**  
Received 3 July 2016  
Received in revised form 22 September 2016  
Accepted 24 September 2016  
Available online xxx

**Keywords:**  
Brazilian conifer  
Micropropagation  
In vitro culture  
Redox metabolism  
Embryonic development

### ABSTRACT

Here we propose a protocol for embryogenic cultures induction, proliferation and maturation for the Brazilian conifer *Podocarpus lambertii*, and investigated the effect of abscisic acid (ABA) and glutathione (GSH) supplementation on the maturation phase. ABA, zeatin (Z) and salicylic acid (SA) endogenous levels were quantified. Number of somatic embryos obtained in ABA-supplemented treatment was significant higher than in ABA-free treatment, showing the relevance of ABA supplementation during somatic embryos maturation. Histological analysis showed the stereotyped sequence of developmental stages in conifer somatic embryos, reaching the late torpedo-staged embryo. GSH supplementation in maturation culture medium improved the somatic embryos number and morphological features. GSH 0 mM and GSH 0.1 mM treatments correlated with a decreased ABA endogenous level during maturation, while GSH 0.5 mM treatment showed constant levels. All treatments resulted in decreased Z endogenous levels, supporting the concept that cytokinins are important during the initial cell division but not for the later stages of embryo development. The lowest SA levels found in GSH 0.5 mM treatment were coincident with early embryonic development, and this treatment resulted in the highest development of somatic embryos. Thus, a correlation between lower SA levels and improved somatic embryo formation can be hypothesized.

© 2016 Published by Elsevier Ltd.

### 1. Introduction

Conifers comprise about 650 species, divided into seven families: Araucariaceae, Cephalotaxaceae, Cupressaceae, Pinaceae, Podocarpaceae, Sciadopityaceae and Taxaceae. Some species of this group are the largest and oldest land organisms on the planet [1]. Among several families of existing conifers, Podocarpaceae stands out as the most diverse family, which contains 18 genera and 173 species and is represented by trees and shrubs used for timber and ornamental purposes, mainly distributed in Southern Hemisphere [2].

*Podocarpus lambertii* Klotzsch ex Endl. (Podocarpaceae) is native from South and Southeastern Brazil and restricted areas of Northwestern Argentina, naturally occurring in the Atlantic Forest Biome [3], which is considered one of the 25 biodiversity hotspots of the world [4]. According to the IUCN (International Union for Conservation of Nature and Natural Resources – www.iucnredlist.org), *P. lambertii* is near threatened, with declining population. Biotechnological tools such as somatic embryogenesis (SE), have potential for clonal propagation and *ex situ* conservation of endangered plant species, especially conifers [5–7].

SE is the developmental reprogramming of somatic cells toward the embryogenic pathway, and forms the basis of cellular totipotency

in higher plants [8]. SE involves dedifferentiation of a nonzygotic cell and subsequent redifferentiation, resulting on the long term in the production of all cell types characteristic of the mature plant. These features make SE an efficient model system for the study of morphological, physiological, molecular and biochemical aspects that occur during the initiation and development in higher plants [8,9].

SE is affected by many factors, such as genotype, culture medium composition, plant growth regulators, gelling agent, type of explant, stress, and light [10]. However, among all these factors, plant growth regulators appear to play the most crucial role in this morphogenetic route [11]. Different molecules play regulatory roles in stress signaling, which is essential for SE induction and control, including those with hormonal activity [12], such as abscisic acid (ABA), zeatin (Z), and salicylic acid (SA). Specifically, ABA is reported to influence some aspects of SE, particularly the phase of somatic embryo maturation, during which it regulates the synthesis and deposition of storage compounds, induces desiccation tolerance and generates somatic embryo dormancy or quiescence [13]. Hence, efforts must be made to obtain a deeper understanding of endogenous hormones as embryogenic potential inducers and regulators of plant embryonic development.

During the last decade, protocols for SE in conifers have undergone rapid and significant progress [14]. However, somatic embryo maturation still a challenging step toward efficient obtainment of uniform and consistent mature somatic embryos [15]. A picture is emerging that shows that optimal early-stage somatic embryo devel-

\* Corresponding author.  
Email address: miguel.guerra@ufsc.br (M.P. Guerra)



## Glutathione and abscisic acid supplementation influences somatic embryo maturation and hormone endogenous levels during somatic embryogenesis in *Podocarpus lambertii* Klotzsch ex Endl.

Hugo Pacheco de Freitas Fraga<sup>a</sup>, Leila do Nascimento Vieira<sup>a</sup>, Catarina Corrêa Puttkammer<sup>a</sup>, Henrique Pessoa dos Santos<sup>b</sup>, Julio de Andrade Garighan<sup>b</sup>, Miguel Pedro Guerra<sup>a, \*</sup>

<sup>a</sup> Laboratório de Fisiologia do Desenvolvimento e Genética Vegetal, Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Florianópolis, SC, 88034-001, Brazil

<sup>b</sup> Laboratório de Fisiologia Vegetal, Empresa Brasileira de Pesquisa Agropecuária, Embrapa Uva e Vinho, Bento Gonçalves, RS, 95700-000, Brazil

### ARTICLE INFO

#### Article history:

Received 3 July 2016

Received in revised form 22 September 2016

Accepted 24 September 2016

Available online xxx

#### Keywords:

Brazilian conifer  
Micropropagation  
*In vitro* culture  
Redox metabolism  
Embryonic development

### ABSTRACT

Here we propose a protocol for embryogenic cultures induction, proliferation and maturation for the Brazilian conifer *Podocarpus lambertii*, and investigated the effect of abscisic acid (ABA) and glutathione (GSH) supplementation on the maturation phase. ABA, zeatin (Z) and salicylic acid (SA) endogenous levels were quantified. Number of somatic embryos obtained in ABA-supplemented treatment was significant higher than in ABA-free treatment, showing the relevance of ABA supplementation during somatic embryos maturation. Histological analysis showed the stereotyped sequence of developmental stages in conifer somatic embryos, reaching the late torpedo-staged embryo. GSH supplementation in maturation culture medium improved the somatic embryos number and morphological features. GSH 0 mM and GSH 0.1 mM treatments correlated with a decreased ABA endogenous level during maturation, while GSH 0.5 mM treatment showed constant levels. All treatments resulted in decreased Z endogenous levels, supporting the concept that cytokinins are important during the initial cell division but not for the later stages of embryo development. The lowest SA levels found in GSH 0.5 mM treatment were coincident with early embryonic development, and this treatment resulted in the highest development of somatic embryos. Thus, a correlation between lower SA levels and improved somatic embryo formation can be hypothesized.

© 2016 Published by Elsevier Ltd.

### 1. Introduction

Conifers comprise about 650 species, divided into seven families: Araucariaceae, Cephalotaxaceae, Cupressaceae, Pinaceae, Podocarpaceae, Sciadopityaceae and Taxaceae. Some species of this group are the largest and oldest land organisms on the planet [1]. Among several families of existing conifers, Podocarpaceae stands out as the most diverse family, which contains 18 genera and 173 species and is represented by trees and shrubs used for timber and ornamental purposes, mainly distributed in Southern Hemisphere [2].

*Podocarpus lambertii* Klotzsch ex Endl. (Podocarpaceae) is native from South and Southeastern Brazil and restricted areas of Northwestern Argentina, naturally occurring in the Atlantic Forest Biome [3], which is considered one of the 25 biodiversity hotspots of the world [4]. According to the IUCN (International Union for Conservation of Nature and Natural Resources – www.iucnredlist.org), *P. lambertii* is near threatened, with declining population. Biotechnological tools such as somatic embryogenesis (SE), have potential for clonal propagation and *ex situ* conservation of endangered plant species, especially conifers [5–7].

SE is the developmental reprogramming of somatic cells toward the embryogenic pathway, and forms the basis of cellular totipotency

in higher plants [8]. SE involves dedifferentiation of a nonzygotic cell and subsequent redifferentiation, resulting on the long term in the production of all cell types characteristic of the mature plant. These features make SE an efficient model system for the study of morphological, physiological, molecular and biochemical aspects that occur during the initiation and development in higher plants [8,9].

SE is affected by many factors, such as genotype, culture medium composition, plant growth regulators, gelling agent, type of explant, stress, and light [10]. However, among all these factors, plant growth regulators appear to play the most crucial role in this morphogenetic route [11]. Different molecules play regulatory roles in stress signaling, which is essential for SE induction and control, including those with hormonal activity [12], such as abscisic acid (ABA), zeatin (Z), and salicylic acid (SA). Specifically, ABA is reported to influence some aspects of SE, particularly the phase of somatic embryo maturation, during which it regulates the synthesis and deposition of storage compounds, induces desiccation tolerance and generates somatic embryo dormancy or quiescence [13]. Hence, efforts must be made to obtain a deeper understanding of endogenous hormones as embryogenic potential inducers and regulators of plant embryonic development.

During the last decade, protocols for SE in conifers have undergone rapid and significant progress [14]. However, somatic embryo maturation still a challenging step toward efficient obtainment of uniform and consistent mature somatic embryos [15]. A picture is emerging that shows that optimal early-stage somatic embryo devel-

\* Corresponding author.

Email address: miguel.guerra@ufsc.br (M.P. Guerra)

opment occurs in the presence of a reducing environment while a shift to an oxidizing environment is required for late-stage development [16,17].

Glutathione (GSH) is a thiol tripeptide formed by glutamic acid, cysteine and glycine ( $\gamma$ -glu-Cys-gly) that can be found in most eukaryotic and prokaryotic cells [18]. In several plant species, the influence of GSH on cell division and differentiation has been reported [19,20]. In *Picea glauca* SE GSH supplementation in the culture medium showed beneficial effects on quantity and morphological features of somatic embryos [19]. Similarly, Vieira et al. [21] reported an increased pro-embryos formation and development in *Araucaria angustifolia* embryogenic cultures subjected to maturation with GSH supplementation, with a strong relationship between GSH addition and nitric oxide levels.

This study provides new insights into SE maturation of *P. lambertii* under a reducing environment caused by GSH supplementation and its consequences in the endogenous hormone levels. Our original approach consists in evaluate the effects of ABA and GSH supplementation during *P. lambertii* somatic embryo development and maturation. GSH supplementation effects on ABA, Z and SA endogenous levels were also investigated. As a consequence, a protocol for embryogenic cultures induction, proliferation and maturation of this Brazilian native conifer is proposed for the first time. Here, we demonstrate remarkable differences in somatic embryos number obtained during maturation with different levels of ABA supplementation. GSH-supplemented treatments indicated improved number and more developed somatic embryos, compared to GSH-free treatment. GSH supplementation affected ABA endogenous levels during embryo maturation, and a correlation between lower SA levels and improved somatic embryo formation could be hypothesized.

## 2. Materials and methods

### 2.1. Somatic embryogenesis induction and proliferation

Immature seeds bearing early globular-staged zygotic embryos were collected in January 2014, from a *P. lambertii* open-pollinated natural population in Lages, Santa Catarina – Brazil (latitude 27°49'0", longitude 50°19'35", altitude 930 m). The seeds were submitted to disinfection procedures with 70% ethanol for 1 min and sodium hypochlorite 1.5% (v/v) for 15 min, followed by a triple-washed with autoclaved distilled water. Zygotic embryos were excised from seeds and inoculated in Petri dishes containing 25 ml of induction culture medium, sealed with PVC-film.

The induction culture medium consisted of MSG macro- and micro-salts [22] supplemented with BM vitamins [23], L-glutamine (1.46 g l<sup>-1</sup>), myo-inositol (0.1 g l<sup>-1</sup>), Phytigel® (2 g l<sup>-1</sup>) and sucrose (30 g l<sup>-1</sup>). The pH of culture medium was adjusted to 5.8 and autoclaved at 121 °C, 1.5 atm for 15 min. All the cultures were maintained in a growth room in the absence of light at temperature of 22 ± 2 °C.

After 30 days induction, the embryogenic cultures (EC) were subcultured in Petri dishes containing 25 ml of the same culture medium composition described for EC induction. Subcultures were made every 15 days for 4 cycles in gelled culture medium for the EC scale-up. Subsequently EC were transferred for proliferation in liquid culture medium (cell suspension) with the same composition as described above, without the gelling agent. The cell suspension was established with 500 mg of EC in 250 ml Erlenmeyer flasks containing 50 ml of liquid culture medium, kept in dark conditions with perma-

nent agitation (90 rpm) in orbital shaker at temperature of 22 ± 2 °C. Subcultures were also made every 15 days for 4 cycles to EC scale-up.

### 2.2. ABA supplementation experiments during maturation phase II

The EC maintained in proliferation cycles in cell suspension were used in different maturation treatments in order to evaluate the ABA optimal concentration for *P. lambertii* somatic embryos obtainment. About 100–200 mg FW of EC suspended in 2 ml of proliferation culture medium was pipetted over a 70-mm diameter filter paper disk in a Büchner funnel. The drained and dispersed EC was then cultured on 90-mm diameter Petri dishes containing 25 ml of maturation phase I (MPI) culture medium. This culture medium consisted of MSG macro- and micro-salts supplemented with BM vitamins, L-glutamine (1.46 g l<sup>-1</sup>), myo-inositol (0.1 g l<sup>-1</sup>), maltose (50 g l<sup>-1</sup>), polyethylene glycol 4000 (PEG) (100 g l<sup>-1</sup>), activated charcoal (2 g l<sup>-1</sup>) and gelled with Phytigel® (3 g l<sup>-1</sup>). The cultures were incubated for 30 days with 16 h photoperiod, with a low light intensity of 5–10 μmol m<sup>-2</sup> s<sup>-1</sup> provided by cool-White OSRAM™ fluorescent lamps, at 22 ± 2 °C, and then transferred to the maturation phase II (MPII) culture medium.

MPII culture medium consisted of the same MPI culture medium composition plus different ABA concentrations (0, 25 and 75 μM). The ABA stock solution was filter sterilized and added to the culture medium after autoclaving. The cultures were incubated for 30 days with 16 h photoperiod, with a low light intensity of 5–10 μmol m<sup>-2</sup> s<sup>-1</sup>, at 22 ± 2 °C. The number of somatic embryos obtained was counted manually with the help of a stereo microscope at 3 x magnification after 15 and 30 days inoculation in MPII culture medium. Cultures monitoring was carried out in Olympus SZH10 Zoom stereo microscope equipped with a computer-controlled digital camera (DP71, Olympus Center Valley, PA, USA).

The experimental design was completely randomized with five replications, and the experimental unit consisted of a Petri dish containing EC. Data were submitted to analysis of variance (ANOVA), followed by Student-Newman-Keuls (SNK) mean separation test ( $p < 0.05$ ), using Statistica® 6.0 for Windows version 8.0.

### 2.3. Light microscopy analysis

Light microscopy technique was used to monitor the development sequence of individually selected somatic embryos derived from the best ABA-supplemented maturation treatment after 0, 15 and 30 days culture on MPII.

Representative samples of *P. lambertii* somatic embryos in different developmental stages were collected and fixed in paraformaldehyde (2.5%) in sodium phosphate buffer 0.2 M (pH 7.2) for 24 h at 4 °C. The samples were then washed three times in buffer without fixative and gradually dehydrated in graded ethanol series (30–100%). The samples were embedded in methacrylate resin (Leica HistoResin®) according to manufacturer's instructions. Sections of 5–7 μm, obtained using a rotary microtome (Slee Technik®), were allowed to adhere to histological slides at 42 ± 2 °C, using few drops of water. After water evaporation, sections were stained with 1% toluidine blue in an aqueous solution of 1% Borax, pH 9 [24], and relevant aspects have been identified and photographed using an inverted microscope (Olympus IX81), equipped with a computer-controlled digital camera.

## 2.4. Glutathione supplementation experiments during MPI

In order to evaluate the effect of different GSH levels supplemented to MPI culture medium, EC maintained in proliferation cycles in cell suspension were used. The same procedures described above were performed, and EC was cultured on 90-mm diameter Petri dishes containing 25 ml of MPI culture medium, and the Petri dishes sealed with PVC-film. This culture medium consisted of the same MPI medium composition described above plus different GSH concentrations (0, 0.1 and 0.5 mM). Cultures were incubated for 30 days with 16 h photoperiod, with a low light intensity of  $5\text{--}10 \mu\text{mol m}^{-2} \text{s}^{-1}$ , at  $22 \pm 2 \text{ }^\circ\text{C}$ , and then transferred to the MPIO culture medium.

MPIO culture medium consisted of the same MPI culture medium composition without GSH and plus the best ABA concentration obtained in the previous experiment. Cultures were incubated for 20 days with 16 h photoperiod, with a low light intensity of  $5\text{--}10 \mu\text{mol m}^{-2} \text{s}^{-1}$ , at  $22 \pm 2 \text{ }^\circ\text{C}$ . The number of somatic embryos obtained was evaluated after 0, 10 and 20 days inoculation in MPIO culture medium. Cultures monitoring was carried out in stereo microscope equipped with a computer-controlled digital camera.

The experimental design was completely randomized with five replications, and the experimental unit consisted of a Petri dish containing EC. Data were submitted to ANOVA, followed by SNK mean separation test ( $p < 0.05$ ), using Statistica<sup>®</sup> 6.0 for Windows version 8.0.

Samples of EC containing somatic embryos obtained from different MPI treatments after 0, 10 and 20 days inoculation in MPIO culture medium were collected for ABA, Z and SA quantification.

## 2.5. ABA, z and SA quantification

The sample extraction and analysis were performed according to an adapted protocol from Müller and Munné-Bosch [25] and Dobrev and Vankova [26]. Lyophilized samples were weighed ( $\sim 20 \text{ mg DW}$ ) in 2 ml microtubes and ground with 800  $\mu\text{l}$  of extraction solvent (methanol:water:formic acid 75:20:5, v/v). Then, the microtubes were kept in  $-20 \text{ }^\circ\text{C}$  overnight and then extracted for 25 min at  $4 \text{ }^\circ\text{C}$  in an ultrasonic bath (40 kHz frequency), followed by centrifugation at  $1,500 \times g$  for 10 min at  $4 \text{ }^\circ\text{C}$ . The pellet was re-extracted 3 more times with 400  $\mu\text{l}$  of extraction solvent and sonicated at the same condition. After the last centrifugation, supernatants were combined, dried in a vacuum concentrator, resuspended in 75  $\mu\text{l}$  of methanol and filtered through a 0.22  $\mu\text{m}$  PTFE filter. Samples were then analyzed by LC-MS/MS.

The system consisted of an Acquity UPLC<sup>™</sup> System (Waters, USA) quaternary pump equipped with an autosampler. An Acquity UPLC BEH C18 (Waters, USA) column ( $2.1 \times 50 \text{ mm}$ ,  $1.7 \mu\text{m}$ ) was used. The mobile phase in the chromatographic separation consisted of a binary mixture of eluent A (0.05% glacial acetic acid in water) and eluent B (0.05% glacial acetic acid in acetonitrile). The gradient consisted of 1% to 100% B in 2.2 min, and kept at 100% B up to 2.8 min at the flow rate of  $0.3 \text{ ml min}^{-1}$ , with column temperature of  $40 \text{ }^\circ\text{C}$ . An injection volume of 5  $\mu\text{l}$  was applied in all analyses. The tandem MS analyses were carried out on a Waters Xevo<sup>™</sup> triple quadrupole mass spectrometer system (MS/MS) equipped with an ESI interface and adjusted with the following conditions: capillary voltage, 2.5 kV; source temperature,  $150 \text{ }^\circ\text{C}$ ; desolvation temperature,  $400 \text{ }^\circ\text{C}$ ; desolvation gas flow,  $800 \text{ l h}^{-1}$ ; cone gas flow,  $20 \text{ l h}^{-1}$ . Multiple reaction monitoring (MRM) mode was used and the para-

eters of MS/MS detection were optimized to each hormone. Analysis of Z was performed in positive ionization ( $\text{ESI}^+$ ,  $220 > 136 \text{ m/z}$ , cone 30 V, collision 17 V), while ABA ( $263 > 153 \text{ m/z}$ , cone 40 V, collision 20 V) and SA ( $137 > 93 \text{ m/z}$ , cone 34 V, collision 18 V) were performed in negative ionization ( $\text{ESI}^-$ ).

Accurately weighed solid portions of ABA (A1049), SA (S5922) and Z (Z0164) standards (Sigma-Aldrich) were dissolved in methanol to prepare  $1 \text{ mg ml}^{-1}$  of stock solutions. All stock solutions were stored under darkness at  $-20 \text{ }^\circ\text{C}$ . Working solutions were prepared from stock solutions with serially dilution in methanol immediately before use. The standard curve, with the concentration sequence of 5, 10, 50, 100, 200, 400, 800, 1600 and  $3200 \text{ ng ml}^{-1}$ , was prepared in three independent dilutions in the black matrix (methanol) with analysis/quantification in LC-MS/MS in triplicate. TargetLynx<sup>™</sup> software (Waters, USA) was used for quantification, with limit of detection (LOD) greater than 3, and the limit of quantification (LOQ) greater than 10. To determine the recovery efficiency and matrix effect, as described by Truffelli et al. [27],  $100 \text{ ng ml}^{-1}$  of standards were spiked in each sample at the beginning of extraction. In parallel, samples were extracted without spiking. The recovery and the matrix effect were determined by comparing the peak areas of the analytical standard spiked before and after extraction.

Data obtained was subjected to one-way analysis of variance (ANOVA) to test the significance of variations in ABA, SA and Z endogenous level of EC containing somatic embryos during maturation. For Post Hoc mean result comparisons, Student–Newman–Keuls (SNK) method ( $p < 0.05$ ) was performed by Statistica<sup>®</sup> 6.0 for Windows version 8.0.

## 3. Results and discussion

### 3.1. ABA supplementation enhances somatic embryos formation and development

Significant differences were found in somatic embryos number obtained during MPIO treatments, with different levels of ABA supplementation (Fig. 1). At day 15 of maturation, 37 somatic embryos/Petri dish were observed in ABA-free treatment. In the same evaluation time, improved somatic embryos formation was observed in ABA-supplemented treatments. At day 30 of MPIO, the best treatment was ABA  $75 \mu\text{M}$ , followed by ABA  $25 \mu\text{M}$  and ABA  $0 \mu\text{M}$ . The improved number of somatic embryos formed in ABA-supplemented

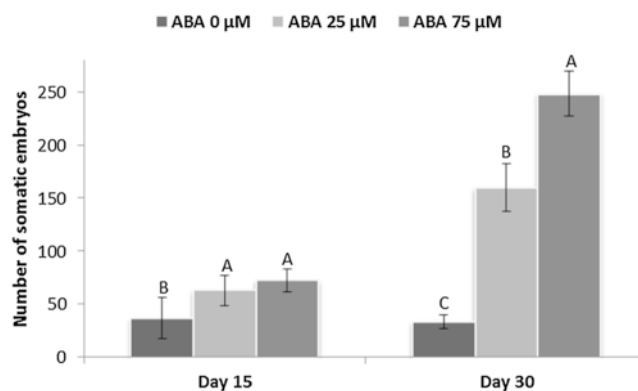


Fig. 1. Somatic embryos number after 15 and 30 days in maturation phase II culture medium with different ABA concentrations (0, 25 and  $75 \mu\text{M}$ ). Mean values  $\pm$  standard deviation. Different letters between treatments indicate significant differences at the same evaluation time according to the SNK test ( $p < 0.05$ ).



treatments highlights the relevance of ABA supplementation during somatic embryos maturation process of *P. lambertii*.

Morphological analysis also demonstrated more organized and developed somatic embryos formation in ABA-supplemented maturation treatments (Fig. 2). After 30 days MPII, somatic embryos in early torpedo developmental stage were found, especially in ABA-supplemented maturation treatments (Fig. 2B,C).

ABA plays a key role in many plant developmental processes, including the promotion of seed desiccation tolerance, maturation of embryos and seed development [28]. ABA also acts as a controlling factor of germination and dormancy in somatic embryos, and is generally used to induce somatic embryos into a quiescent state during plant tissue culture [28]. Furthermore, ABA not only promotes the transition of somatic embryos from the proliferation to the maturation phase, but it has also been used to enhance embryo morphological features by increasing desiccation tolerance and preventing precocious germination [28–30].

The yield of mature somatic embryos of Norway spruce on ABA-containing medium was increased up to 10-fold when a pretreatment of 1–9 days with this plant growth regulator was applied [31]. Zhang et al. [32] reported the role of exogenously applied ABA during different stages of *Arabidopsis thaliana* embryo development, further suggesting that the promotion of somatic embryos development is ABA-dependent. In the present study, ABA supplementation also improved somatic embryos morphological features and number, in agreement with these previous reports.

Production of synchronous mature somatic embryos that undergo a period of arrest during development is a prerequisite for a successful somatic embryogenesis protocol [14]. Larch somatic embryos cultured on ABA-supplemented culture medium were synchronous and developed coherently into late cotyledonary embryos, while somatic embryos obtained on ABA-free culture medium were asynchronous and with different morphological features [33]. Here, we did not quantitatively evaluate embryos synchrony, but we observed the presence of somatic embryos in higher developmental stages in ABA-supplemented treatments.

Thus, ABA supplementation in the culture medium during MPII appears to substantially increase the number and morphological features of *P. lambertii* somatic embryos, and should be performed in order to promote their further development.

### 3.2. Light microscopy analysis of somatic embryos

Globular somatic embryos at the onset of polarization showing a well-delimited protoderm were the first clearly distinguishable stage of somatic embryo histodifferentiation (Fig. 3A). A layer of embryonal tube cells and suspensor-like cells was also evident, which appeared to be more vacuolated as compared to cells of the embryonic

head. Subsequent somatic embryo development included their elongation, development of procambium and shoot meristem differentiation, reaching the early torpedo-staged somatic embryo (Fig. 3B). Finally, an increased embryo elongation was observed, which became sharper, reaching the late torpedo-staged somatic embryo (Fig. 3C). Morphological features of the respective somatic embryo stages used in the histological analysis are indicated in Fig. 3D (arrows).

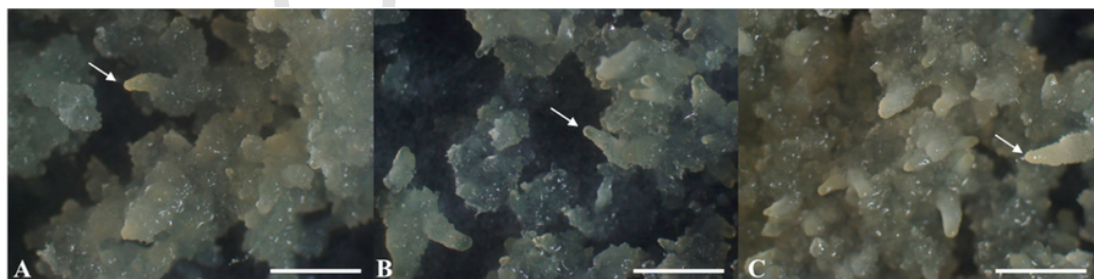
Very similar histological features were also observed during somatic embryo development in other Brazilian non-pinaceae conifer, *Araucaria angustifolia* [7]. These authors reported a conspicuous presence of intercellular spaces, especially in torpedo-staged somatic embryo, unlike the results found in the present study. The absence of these intercellular spaces in *P. lambertii* somatic embryos may indicate a better embryonic development. Poorly developed shoot apical meristems, disrupted by the presence of large intercellular air spaces, have been reported in many genera of conifers, as reviewed by Stasolla and Yeung [29].

Somatic embryos in the first stages of development of *A. angustifolia* were characterized by individualized structures composed of two polarized regions: a dense globular embryonic head connected to a suspensor region [34]. The embryonic head was composed by embryogenic cells, and the suspensor region was composed by suspensor cells [34]. These authors also described a transitional region between these both regions. Our results also indicated these features, especially in the globular-staged somatic embryo.

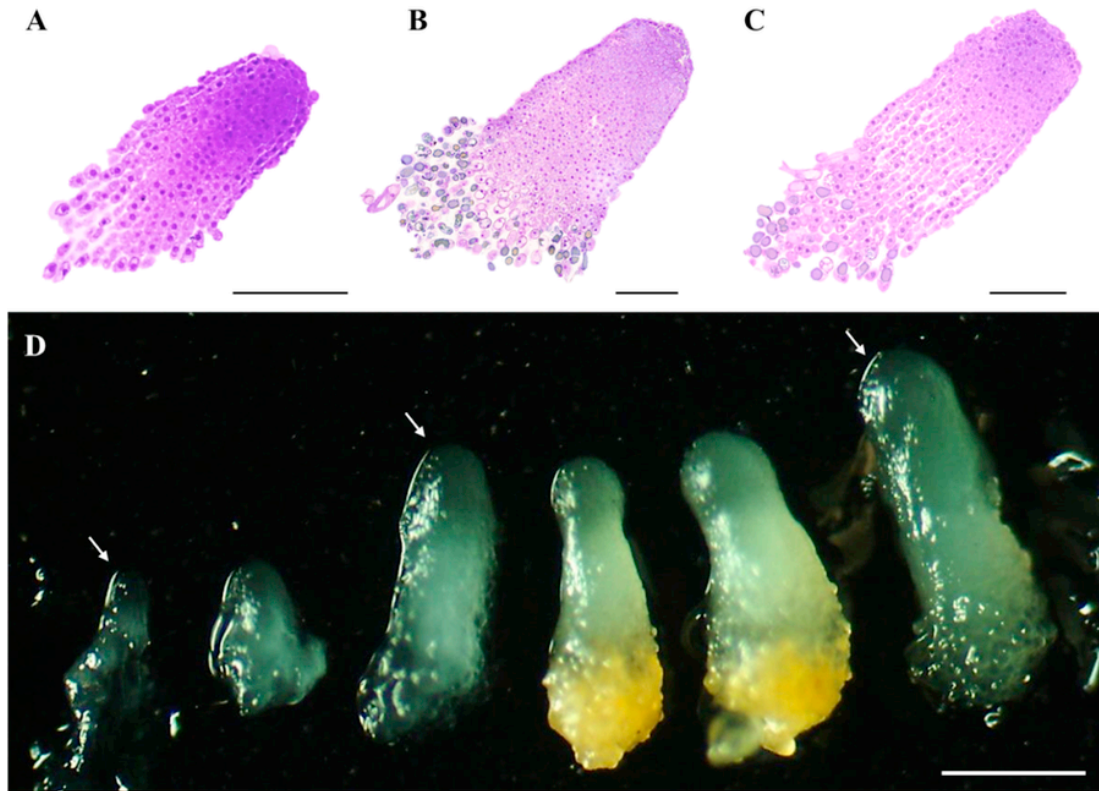
In our study, during the transition of early torpedo- to late torpedo-staged somatic embryos, we observed an enlargement of embryonic head and an improved organization of suspensor structure (Fig. 3C). Similarly, Morel et al. [15] reported that meristematic centers gradually enlarged, and suspensor cells became well organized, comprising a number of long narrow cells after 3 weeks maturation of *Pinus pinaster* EC. Histological features found in the present study are also in agreement to histological analysis performed in distinct developmental stages of *Picea abies* somatic embryos, a Pinaceae conifer, which showed similar pattern of ultrastructural organization [35]. However, the subsequent stages of torpedo-staged somatic embryos were not achieved with the maturation treatment tested in the present study.

### 3.3. Different supplemented glutathione levels improve somatic embryos maturation

The effect of different glutathione levels supplemented to maturation culture MPI on *P. lambertii* somatic embryos maturation was investigated, and pronounced differences in embryo number and morphological features were observed. At inoculation time in MPII culture medium (after 30 days on MPI), treatment with GSH 0.5 mM indicated the highest number of somatic embryos/Petri dish, followed



**Fig. 2.** Morphological features of embryogenic cultures containing somatic embryos (arrows) derived from different maturation phase II treatments supplemented with ABA 0  $\mu$ M (A), ABA 25  $\mu$ M (B) and ABA 75  $\mu$ M (C) after 30 days in culture. Bar: 2.0 mm.



**Fig. 3.** Histological analysis of *P. lambertii* somatic embryos. A – Early globular somatic embryo; B – Early torpedo-staged somatic embryo (LG) showing a layer of embryonal tube cells and suspensor-like cells. Note the presence of more vacuolated cells in the basal part of the embryo. C – Late torpedo-staged somatic embryo showing meristematic cells in the apical part with embryonal tube cells in the middle part until the basal part consisting of suspensor cells. D – Morphological aspects of different somatic embryos developmental stages. Arrows indicate the early globular, early torpedo and late torpedo-staged somatic embryos. Bar figures A, B and C: 200  $\mu$ m. Bar figure D: 1.0 mm.

by GSH 0.1 mM and GSH 0 mM (Fig. 4). The same scenario was observed in day 10 MPII, with 659, 467 and 299.2 somatic embryos/Petri dish in GSH 0.5, GSH 0.1 and GSH 0 mM treatments, respectively. However, no statistical difference was found between treatments in day 20 MPII. Nevertheless, it is important to emphasize that GSH-supplemented treatments indicated more developed somatic embryos as compared to GSH-free treatment in this evaluation time (Fig. 5).

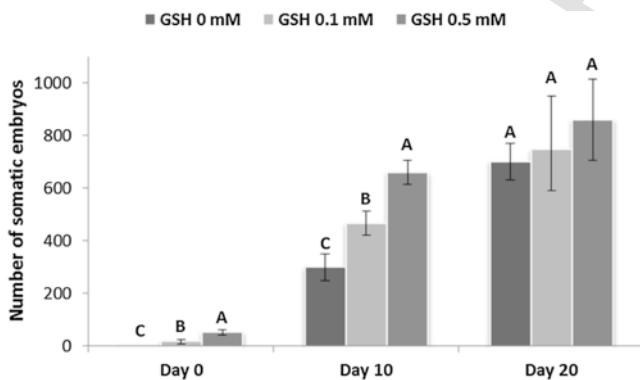
Glutathione is an important antioxidant that protects cells against oxidative stress and functions in biosynthetic pathways, antioxidant

biochemistry and redox homeostasis [36]. Changes in the glutathione redox state play an important role in a number of biological processes where they affect many cell activities and functions, including somatic embryo yield and morphological features [16]. Pullman et al. [17] found that culture medium supplementation with GSH increased early-stage somatic embryo growth and embryogenic tissue culture initiation for cultures of *Pinus taeda* and *Pinus menziesii*.

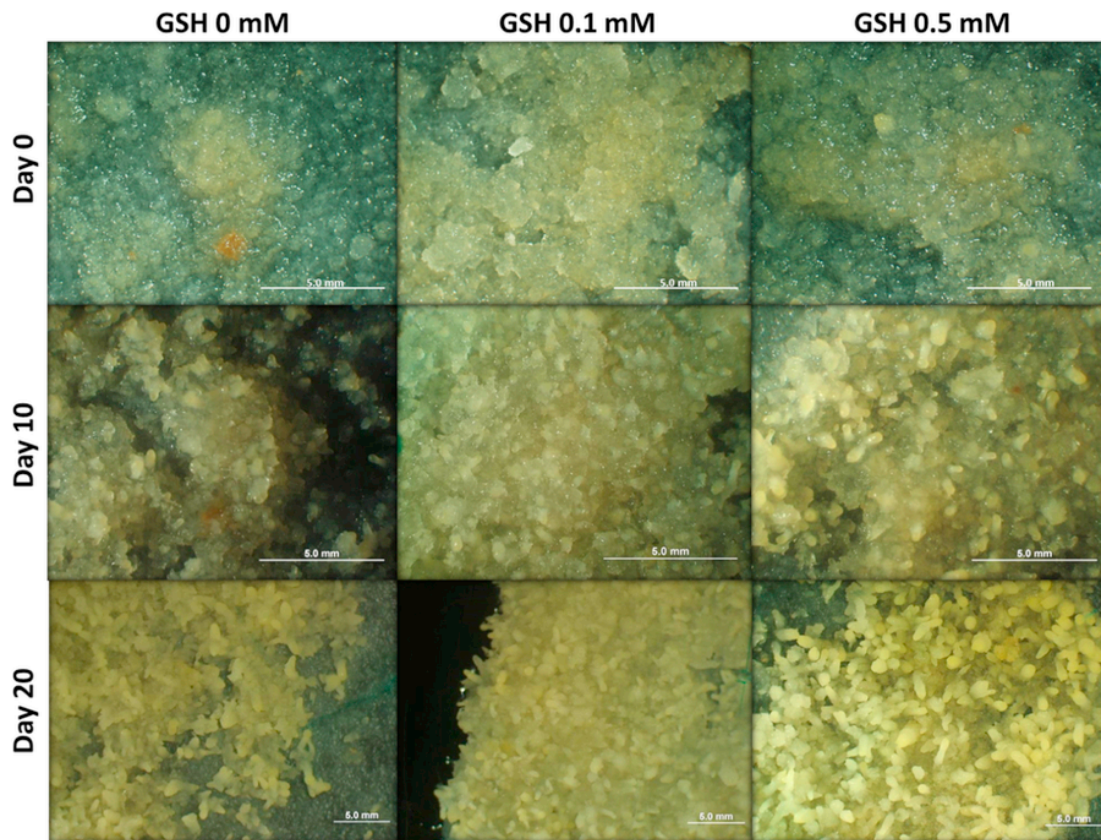
Belmonte and Yeung [37] also reported an increased total number of somatic embryos in *Picea glauca* in response to the supplementation of 0.1 mM GSH in the ABA-containing maturation culture medium. These authors also observed a significantly higher number of somatic embryos that were able to develop to the mature cotyledonary stage. Our study indicated similar results, with an improvement in mature somatic embryos formation in GSH-supplemented treatments (Fig. 5).

Glutathione supplementation at low levels (0.01 and 0.1 mM) increased the number of *A. angustifolia* early somatic embryos, with 35% of total pro-embryogenic mass indicating polarization after 7 days culture, in contrast to GSH-free treatment (8%). However, EC maintenance for longer than 7 days in an environment with high redox potential proved to be deleterious to the early somatic embryos development, causing the somatic embryos oxidation and death [21].

A reduced glutathione environment during the induction of the embryogenic process increases the number of immature somatic embryos, whereas the imposition of an oxidized environment ensures the proper completion of the developmental program [16]. In the present study, the somatic embryos obtained in GSH-supplemented treatments reached advanced developmental stages. Thus, the manipulation of GSH/GSSG (glutathione disulfide) ratio, transferring the



**Fig. 4.** Somatic embryos number after 0, 10 and 20 days in maturation phase II culture medium derived from treatments supplemented with different GSH concentrations (0, 0.1 and 0.5 mM) during the maturation phase I. Mean values  $\pm$  standard deviation. Different letters between treatments indicate significant differences at the same evaluation time according to the SNK test ( $p < 0.05$ ).



**Fig. 5.** Morphological features of embryogenic cultures containing somatic embryos derived from different maturation phase I treatments supplemented with GSH 0, 0.1 and 0.5 mM after 0, 10 and 20 days in maturation phase II. Bar: 5 mm.

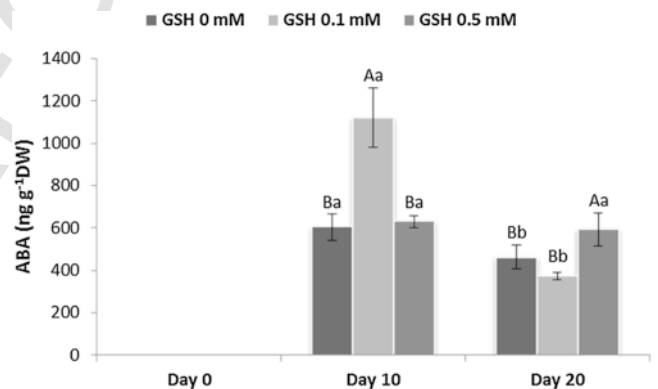
EC to a culture medium supplemented with GSSG during the final maturation process, may be a suitable strategy to develop a more efficient embryogenic system for *P. lambertii*.

### 3.4. GSH supplementation affects endogenous levels of ABA, Z and SA during embryos maturation

Endogenous levels of ABA, Z and SA from EC submitted to different GSH-supplemented maturation treatments indicated diverse accumulation dynamics (Figs. 6–8). Despite the observed matrix effect in hormone quantification, this influence was constant in all treatments and did not interfere in the results. It was verified an increase of 11.55 fold in ABA and a decrease of 0.66 fold for Z and 0.38 fold for SA.

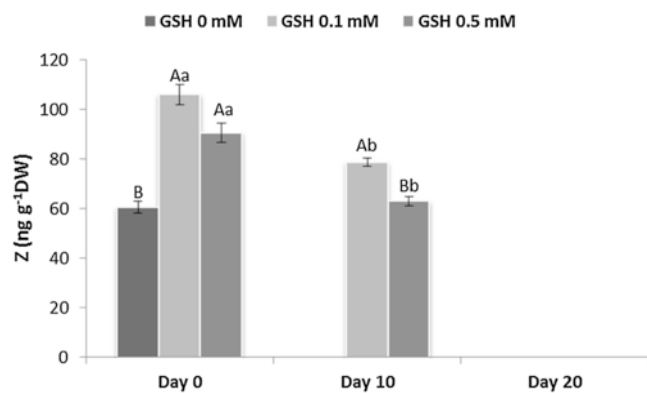
At day 0 MPII, no detectable ABA was observed in any treatment (Fig. 6). After 10 days of MPII, GSH 0.1 mM treatment indicated the highest ABA levels ( $1,120.7 \text{ ng g}^{-1} \text{ DW}$ ), followed by GSH 0.5 mM ( $630 \text{ ng g}^{-1} \text{ DW}$ ) and GSH 0 mM ( $604.2 \text{ ng g}^{-1} \text{ DW}$ ) treatments, which indicated equal levels. In day 20, GSH 0.1 mM and GSH 0 mM treatments indicated the lowest ABA levels ( $373.3$  and  $461.5 \text{ ng g}^{-1} \text{ DW}$ , respectively), and GSH 0.5 mM the highest ( $593.1 \text{ ng g}^{-1} \text{ DW}$ ). Contrasting the evaluation times (days 10 and 20), treatments supplemented with GSH 0 mM and GSH 0.1 mM indicated a decrease during maturation, while GSH 0.5 treatment showed constant ABA levels (Fig. 6).

Typically, ABA endogenous level is low during the initial phases of somatic embryo development, increases during the embryonic growth, and then decreases during the last maturation stages, at the onset of the desiccation period [29]. Several studies have reported

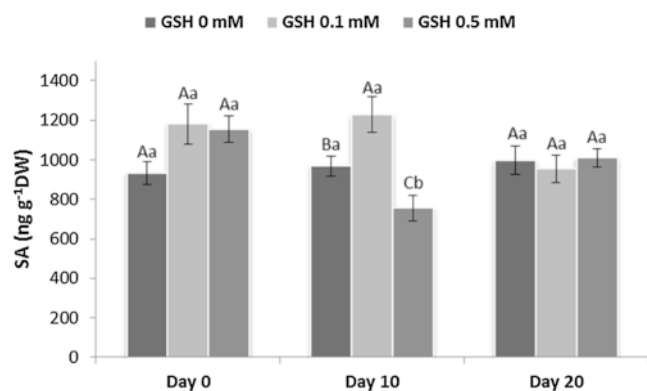


**Fig. 6.** Abscisic acid (ABA) endogenous levels of embryogenic cultures after 0, 10 and 20 days in maturation phase II culture medium derived from treatments supplemented with different GSH concentrations (0, 0.1 and 0.5 mM) during the maturation phase I. Mean values  $\pm$  standard deviation. Different uppercase letters indicate significant differences at the same evaluation time, and different lowercase letters indicate significant differences along the evaluation times within treatments, according to the SNK test ( $p < 0.05$ ). DW: dry weight.

this pattern of ABA accumulation during somatic embryos maturation on *Pinus pinaster* [15], carrot [38], *Medicago falcata* [39], *Camellia sinensis* [40], and *Quercus suber* [41,42]. In the present study, this typical behavior was specially observed on GSH 0.1 mM treatment, which presented a much more expressive decrease in ABA endogenous levels after day 20 in maturation, as compared to the other treatments.



**Fig. 7.** Zeatin (Z) endogenous levels of embryogenic cultures after 0, 10 and 20 days in maturation phase II culture medium derived from treatments supplemented with different GSH concentrations (0, 0.1 and 0.5 mM) during the maturation phase I. Mean values  $\pm$  standard deviation. Different uppercase letters indicate significant differences at the same evaluation time, and different lowercase letters indicate significant differences along the evaluation times within treatments, according to the SNK test ( $p < 0.05$ ). DW: dry weight.



**Fig. 8.** Salicylic acid (SA) endogenous levels of embryogenic cultures after 0, 10 and 20 days in maturation phase II culture medium derived from treatments supplemented with different GSH concentrations (0, 0.1 and 0.5 mM) during the maturation phase I. Mean values  $\pm$  standard deviation. Different uppercase letters indicate significant differences at the same evaluation time, and different lowercase letters indicate significant differences along the evaluation times within treatments, according to the SNK test ( $p < 0.05$ ). DW: dry weight.

Few reports can be found on the literature addressing the glutathione supplementation and ABA endogenous level during somatic embryos maturation. Belmonte et al. [20] reported an improved ABA endogenous level in *Brassica napus* somatic embryos treated with buthionine sulfoximine (BSO), an inhibitor of glutathione *de novo* synthesis. These authors hypothesized that the higher availability and turnover of ABA in BSO-treated embryos may be responsible for the proper execution of all developmental events, resulting in the production of embryos with improved morphological features. Our results also indicate that glutathione supplementation affects ABA levels during embryo maturation; however, further experiments with BSO at the final maturation phase may be better elucidate this relationship.

Regarding Z endogenous levels, at day 0 MPII, GSH-supplemented treatments showed the highest levels as compared to GSH-free treatment (Fig. 7). After 10 days of MPII, GSH 0.1 mM treatment resulted in the highest Z levels ( $78.64 \text{ ng g}^{-1} \text{ DW}$ ), followed by GSH 0.5 mM ( $62.9 \text{ ng g}^{-1} \text{ DW}$ ). No Z was detected in any treatment. In day 20, no Z was detected in any treatment.

There is support for the concept that cytokinins, in general, are important during the initial cell division phase of SE, but not for later stages of embryo development and maturation [43,44]. This suggests that cytokinins may have a major role in cell division, but not in embryo differentiation [30].

Among the few reports regarding Z endogenous levels during somatic embryo maturation, Vágner et al. [45] found that cytokinin levels, including Z, dramatically dropped after ABA supplementation to culture medium during *Picea abies* somatic embryo maturation, and this decrease were correlated to ABA concentration used. In contrast, hybrid larch somatic embryos showed an increased cytokinin level during the maturation period [46]. A similar pattern was also found in larch somatic embryos submitted to maturation in presence activated charcoal, with this increase being dependent on whether the medium was supplemented with charcoal [47].

A correlation between decreased Z levels and embryogenic potential has also been reported. Pérez-Jiménez et al. [10] found a higher Z endogenous content in *Prunus persica* non-embryogenic cultures than in embryogenic ones. Similar results were reported in embryogenic genotypes of hazelnut, where Z levels were found [48], as well as in coconut embryogenic cultures [49]. The results of the present work corroborate these findings, where undetectable Z levels were found in more advanced stages of somatic embryos maturation.

Levels of SA were also evaluated, as shown in Fig. 8. At day zero MPII, no differences were observed between treatments. Differently, in day 10 of maturation, GSH 0.1 mM treatment indicated highest levels of SA ( $1,227.2 \text{ ng g}^{-1} \text{ DW}$ ), followed by GSH 0 mM ( $969 \text{ ng g}^{-1} \text{ DW}$ ) and GSH 0.5 mM ( $754.7 \text{ ng g}^{-1} \text{ DW}$ ). Again, no differences were observed at day 20 of maturation. Contrasting the SA levels along the evaluation times, only GSH 0.5 mM treatment indicated significant differences, with a substantial decrease at day 10 of maturation, followed by an increase.

Salicylic acid belongs to the large group of plant phenolics, being ubiquitous in higher plants, and an important signaling molecule involved in plant defense responses to pathogens and abiotic stress, as well as in plant growth and development [50,51]. During SE of *Astragalus adsurgens*, SA supplementation to maturation culture medium significantly enhanced somatic embryos formation [52]. In the same way, picomolar SA concentrations exogenously applied in *Coffea arabica* embryogenic cultures induced cellular growth, and enhanced the number and morphological features of somatic embryos [53]. A plausible explanation may be that SA has a biphasic effect in plants: when SA is at a low concentration it can induce cellular reprogramming of somatic cells into the embryogenic stage, while at a high concentration this reprogramming can be inhibited, inducing another cellular reprogram for cellular viability to be preserved [54].

Results observed in GSH 0.5 mM treatment, in our study, indicated the lowest SA levels at day 10 maturation, period coincident with early embryonic development. This treatment, in the same evaluation time, indicated the highest somatic embryos formation as compared to the other treatments. In this sense, a correlation between lower SA levels and improved somatic embryo formation can be hypothesized, corroborating to these findings.

Kanno et al. [55], investigating the pattern of SA endogenous accumulation during *Arabidopsis thaliana* zygotic embryos development, reported that the SA levels were relatively high at early embryonic stages, decreasing in mid-development and subsequently increasing in late stages. Again, GSH 0.5 mM treatment showed this same accumulation pattern, reinforcing the idea of GSH supplementation at this concentration may be improve the redox status during *P. lambertii* somatic embryo development.

#### 4. Conclusion

Taken together, the results of present study provided consistent data to improve the SE morphogenetic route in non-Pinaceae conifers, as is the case of *P. lambertii*. The central role of ABA in the somatic embryos maturation process of *P. lambertii* was demonstrated, being apparently essential as a maturation promoter. In addition, glutathione supplementation on the MPI culture medium resulted in pronounced differences in embryo number and morphological features. Somatic embryos obtained in GSH-supplemented treatments reached advanced developmental stages; however, the final stages of maturation and conversion could not be achieved. Thus, the manipulation of GSH/GSSG ratio during the final maturation process may be a suitable strategy to develop a more efficient embryogenic system in *P. lambertii*.

Finally, endogenous levels of ABA, Z and SA from EC submitted to different GSH-supplemented maturation treatments indicated diverse accumulation dynamics. Our results indicated that GSH supplementation affects ABA levels during embryo maturation; however, further experiments with BSO at the final maturation phase may better elucidate this relationship. Regarding to Z endogenous levels, all treatments indicated significant decreased levels during the maturation phase, supporting the concept that cytokinins, in general, are important during the initial cell division phase of somatic embryogenesis, but not for the later stages of embryo development and maturation. In addition, a correlation between lower SA levels and improved somatic embryo formation were hypothesized.

#### Authors contribution

Conceived and designed the experiments: HPFF, LNV and MPG; Performed the *in vitro* culture experiments: HPFF, LNV and CCP; Performed the LC-MS/MS quantification and data analysis: HPFF, LNV, JAG and HPS; Contributed reagents/materials/analysis tools: HPS and MPG; Wrote the paper: HPFF, LNV and MPG.

#### Acknowledgements

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 306126/2013-3), and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC 14848/2011-2, 3770/2012, and 2780/2012-4). The authors thank to Dr. Adelar Mantovani and José Antônio Ribas Ribeiro for kindly provide *Podocarpus lambertii* seeds for somatic embryogenesis induction. The authors are also grateful to Dr. Emanuel Maltempi de Souza, Dr. Lauro Souza and Dr. Marcelo Muller-Santos for the important insights about the hormones quantification.

#### References

- [1] M. Ahuja, D. Neale, Evolution of genome size in conifers, *Silvae Genet.* 54 (2005) 126–137.
- [2] T.T. Veblen, J.J. Armesto, B.R. Burns, T. Kitzberger, A. Lara, R. Young, The coniferous forests of south america, *Ecosyst. World Conifer. For.*, Amsterdam, 2005701–725.
- [3] A. Farjon, D. Filer, An Atlas of the World's Conifers: an Analysis of Their Distribution, Biogeography, Diversity and Conservation Status, Brill, Leiden–Boston, 2013.
- [4] N. Myers, R.A. Mittermeier, C.G. Mittermeier, G.A.B. da Fonseca, J. Kent, Biodiversity hotspots for conservation priorities, *Nature* 403 (2000) 853–858, <http://dx.doi.org/10.1038/35002501>.
- [5] M.P. Guerra, V. Silveira, A.L.W. Dos Santos, L.V. Astarita, R.O. Nodari, Somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Kuntze, in: *Somat. Embryog. Woody Plants* (2000) pp. 457–578. [10.1007/978-94-017-3030-3\\_15](https://doi.org/10.1007/978-94-017-3030-3_15).
- [6] K. Klimaszewska, C. Overton, D. Stewart, R.G. Rutledge, Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process, *Planta* 233 (2011) 635–647, <http://dx.doi.org/10.1007/s00425-010-1325-4>.
- [7] H.P.F. Fraga, L.N. Vieira, C.C. Puttkammer, E.M. Oliveira, M.P. Guerra, Time-lapse cell tracking reveals morphohistological features in somatic embryogenesis of *Araucaria angustifolia* (Bert) O. Kuntze, *Trees – Struct. Funct.* 29 (2015) 1613–1623, <http://dx.doi.org/10.1007/s00468-015-1244-x>.
- [8] O. Karami, A. Saidi, The molecular basis for stress-induced acquisition of somatic embryogenesis, *Mol. Biol. Rep.* 37 (2010) 2493–2507, <http://dx.doi.org/10.1007/s11033-009-9764-3>.
- [9] C.X. Zhang, Q. Li, L. Kong, Induction, development and maturation of somatic embryos in Bunge's pine (*Pinus bungeana* Zucc. ex Endl.), *Plant Cell Tissue Organ Cult.* 91 (2007) 273–280, <http://dx.doi.org/10.1007/s11240-007-9294-4>.
- [10] M. Pérez-Jiménez, E. Cantero-Navarro, F. Pérez-Alfocea, I. Le-Disquet, A. Guivarc'h, J. Cos-Terrer, Relationship between endogenous hormonal content and somatic organogenesis in callus of peach (*Prunus persica* L. Batsch) cultivars and *Prunus persica* × *Prunus dulcis* rootstocks, *J. Plant Physiol.* 171 (2014) 619–624, <http://dx.doi.org/10.1016/j.jplph.2014.01.006>.
- [11] I. Ruduś, E. Kepczyńska, J. Kepczyński, Comparative efficacy of abscisic acid and methyl jasmonate for indirect somatic embryogenesis in *Medicago sativa* L., *Plant Growth Regul.* 48 (2006) 1–11, <http://dx.doi.org/10.1007/s10725-005-5136-8>.
- [12] I. Ruduś, E.W. Weiler, E. Kepczyńska, Do stress-related phytohormones, abscisic acid and jasmonic acid play a role in the regulation of *Medicago sativa* L. somatic embryogenesis?, *Plant Growth Regul.* 59 (2009) 159–169, <http://dx.doi.org/10.1007/s10725-009-9399-3>.
- [13] S. Von Arnold, I. Sabala, P. Bozhkov, J. Dyachok, L. Filonova, Developmental pathways of somatic embryogenesis, *Plant Cell. Tissue Organ Cult.* 69 (2002) 233–249, <http://dx.doi.org/10.1023/A:1015673200621>.
- [14] M.A. Lelu-Walter, D. Thompson, L. Harvengt, L. Sanchez, M. Toribio, L.E. Pâques, Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction, *Tree Genet Genomes* 9 (2013) 883–899, <http://dx.doi.org/10.1007/s11295-013-0620-1>.
- [15] A. Morel, C. Teyssier, J.-F. Trontin, K. Eliasova, B. Pesek, M. Beaufour, D. Morabito, N. Boizot, C. Le Mété, L. Belal-Bessai, I. Reymond, L. Harvengt, M. Cadene, F. Corbineau, M. Vágner, P. Label, M.A. Lelu-Walter, Early molecular events involved in *Pinus pinaster* Ait. somatic embryo development under reduced water availability: transcriptomic and proteomic analyses, *Physiol. Plant.* 152 (2014) 184–201, <http://dx.doi.org/10.1111/ppl.12158>.
- [16] C. Stasolla, Glutathione redox regulation of *in vitro* embryogenesis, *Plant Physiol. Biochem.* 48 (2010) 319–327, <http://dx.doi.org/10.1016/j.plaphy.2009.10.007>.
- [17] G.S. Pullman, X. Zeng, B. Copeland-Kamp, J. Crockett, J. Lucrezi, S.W. May, K. Bucalo, Conifer somatic embryogenesis: improvements by supplementation of medium with oxidation-reduction agents, *Tree Physiol.* 35 (2015) 209–224, <http://dx.doi.org/10.1093/treephys/tpu117>.
- [18] C.H. Foyer, G. Noctor, Ascorbate and glutathione: the heart of the redox hub, *Plant Physiol.* 155 (2011) 2–18, <http://dx.doi.org/10.1104/pp.110.167569>.
- [19] M.F. Belmonte, G. Donald, D.M. Reid, E.C. Yeung, C. Stasolla, Alterations of the glutathione redox state improve apical meristem structure and somatic embryo quality in white spruce (*Picea glauca*), *J. Exp. Bot.* 56 (2005) 2355–2364, <http://dx.doi.org/10.1093/jxb/eri228>.
- [20] M.F. Belmonte, S.J. Ambrose, A.R.S. Ross, S.R. Abrams, C. Stasolla, Improved development of microspore-derived embryo cultures of *Brassica napus* cv Topaz following changes in glutathione metabolism, *Physiol. Plant.* 127 (2006) 690–700, <http://dx.doi.org/10.1111/j.1399-3054.2006.00707.x>.
- [21] L. do, N. Vieira, C. Santa-Catarina, H.P. de, F. Fraga, A.L.W. dos Santos, D.A. Steinmacher, P.S. Schlogl, V. Silveira, N. Steiner, E.I.S. Floh, M.P. Guerra, Glutathione improves early somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Kuntze by alteration in nitric oxide emission, *Plant Sci.* 195 (2012) 80–87.
- [22] M.R. Becwar, R. Nagmani, S.R. Wann, Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*), *Can. J. For. Res.* 20 (1990) 810–817, <http://dx.doi.org/10.1139/x90-107>.
- [23] G. Pullman, P. Gupta, Method for reproducing coniferous plants by somatic embryogenesis using adsorbent materials in the development stage media, 1991. U.S. Patent No. 5036007.
- [24] J. Kuo, T. O'Brien, Lignified sieve elements in the wheat leaf, *Planta* 117 (1974) 349–353, <http://dx.doi.org/10.1007/BF00388029>.
- [25] M. Müller, S. Munné-Bosch, Rapid and sensitive hormonal profiling of complex plant samples by liquid chromatography coupled to electrospray ionization tandem mass spectrometry, *Plant Methods.* 7 (2011) 37, <http://dx.doi.org/10.1186/1746-4811-7-37>.

- [26] P.I. Dobrev, R. Vankova, Quantification of abscisic acid, cytokinin, and auxin content in salt-stressed plant tissues, *Methods Mol. Biol.* 913 (2012) 251–261, <http://dx.doi.org/10.1007/978-1-61779-986-0-17>.
- [27] H. Truffelli, P. Palma, G. Famigliani, A. Cappiello, An overview of matrix effects in liquid chromatography-mass spectrometry, *Mass Spectrom. Rev.* 30 (2011) 491–509, <http://dx.doi.org/10.1002/mas.20298>.
- [28] M.K. Rai, N.S. Shekhawat, Harish, A.K. Gupta, M. Phulwaria, K. Ram, U. Jaiswal, The role of abscisic acid in plant tissue culture: a review of recent progress, *Plant Cell Tissue Organ Cult.* 106 (2011) 179–190, <http://dx.doi.org/10.1007/s11240-011-9923-9>.
- [29] C. Stasolla, E.C. Yeung, Recent advances in conifer somatic embryogenesis: improving somatic embryo quality, *Plant Cell. Tissue Organ Cult.* 74 (2003) 15–35, <http://dx.doi.org/10.1023/A:1023345803336>.
- [30] V.M. Jiménez, Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis, *Plant Growth Regul.* 47 (2005) 91–110, <http://dx.doi.org/10.1007/s10725-005-3478-x>.
- [31] P.V. Bozhkov, L.H. Filonova, S. Von Arnold, A key developmental switch during Norway spruce somatic embryogenesis is induced by withdrawal of growth regulators and is associated with cell death and extracellular acidification, *Biotechnol. Bioeng.* 77 (2002) 658–667, <http://dx.doi.org/10.1002/bit.10228>.
- [32] H. Zhang, W. Han, I. De Smet, P. Talboys, R. Loya, A. Hassan, H. Rong, G. Jürgens, J. Paul Knox, M.H. Wang, ABA promotes quiescence of the quiescent centre and suppresses stem cell differentiation in the Arabidopsis primary root meristem, *Plant J.* 64 (2010) 764–774, <http://dx.doi.org/10.1111/j.1365-313X.2010.04367.x>.
- [33] J.H. Zhang, S.G. Zhang, S.G. Li, S.Y. Han, W.F. Li, X.M. Li, L.W. Qi, Regulation of synchronism by abscisic-acid-responsive small noncoding RNAs during somatic embryogenesis in larch (*Larix leptolepis*), *Plant Cell. Tissue Organ Cult.* 116 (2014) 361–370, <http://dx.doi.org/10.1007/s11240-013-0412-1>.
- [34] N. Steiner, F.L. Farias-Soares, É.C. Schmidt, M.L.T. Pereira, B. Scheid, G.D. Rogge-Renner, Z.L. Bouzon, D. Schmidt, S. Maldonado, M.P. Guerra, Toward establishing a morphological and ultrastructural characterization of proembryonic masses and early somatic embryos of *Araucaria angustifolia* (Bert.) O. Kuntze, *Protoplasma* 253 (2016) 487–501, <http://dx.doi.org/10.1007/s00709-015-0827-0>.
- [35] L.H. Filonova, P.V. Bozhkov, S. von Arnold, Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking, *J. Exp. Bot.* 51 (2000) 249–264, <http://dx.doi.org/10.1093/jexbot/51.343.249>.
- [36] G. Noctor, A. Mhamdi, S. Chaouch, Y. Han, J. Neukermans, B. Marquez-Garcia, G. Queval, C.H. Foyer, Glutathione in plants: an integrated overview, *Plant Cell Environ.* 35 (2012) 454–484, <http://dx.doi.org/10.1111/j.1365-3040.2011.02400.x>.
- [37] M.F. Belmonte, E.C. Yeung, The effects of reduced and oxidized glutathione on white spruce somatic embryogenesis, *In Vitro. Cell Dev. Biol. Plant.* 40 (2004) 61–66, <http://dx.doi.org/10.1079/IVP2003483>.
- [38] H. Kamada, H. Harada, Changes in the endogenous level and effects of abscisic acid during somatic embryogenesis of *Daucus carota* L., *Plant Cell Physiol.* 22 (1981) 1423–1429.
- [39] A. Ivanova, M. Velcheva, P. Denchev, A. Atanassov, H.A. van Onckelen, Endogenous hormone levels during direct somatic embryogenesis in *Medicago falcata*, *Physiol. Plant.* 92 (1994) 85–89, <http://dx.doi.org/10.1034/j.1399-3054.1994.920112.x>.
- [40] P. Sharma, S. Pandey, A. Bhattacharya, P.K. Nagar, P.S. Ahuja, ABA associated biochemical changes during somatic embryo development in *Camellia sinensis* (L.) O. Kuntze, *J. Plant Physiol.* 161 (2004) 1269–1276, <http://dx.doi.org/10.1016/j.jplph.2004.01.015>.
- [41] M. Pérez, M. Viejo, M. LaCuesta, P. Toorop, M.J. Cañal, Epigenetic and hormonal profile during maturation of *Quercus suber* L. somatic embryos, *J. Plant Physiol.* 173 (2015) 51–61, <http://dx.doi.org/10.1016/j.jplph.2014.07.028>.
- [42] G. García-Martín, J.A. Manzanera, M.E. González-Benito, Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos, *Plant Cell Tissue Organ Cult.* 80 (2005) 171–177, <http://dx.doi.org/10.1007/s11240-004-1056-y>.
- [43] T. Fujimura, A. Komamine, Mode of action of 2 4-D and zeatin on somatic embryogenesis in a carrot cell suspension culture, *Zeitschrift Für Pflanzenphysiologie* 99 (1980) 1–8, [http://dx.doi.org/10.1016/S0044-328X\(80\)80106-1](http://dx.doi.org/10.1016/S0044-328X(80)80106-1).
- [44] V.M. Jiménez, Regulation of in vitro somatic embryogenesis with emphasis on the role of endogenous hormones, *Rev. Bras. Fisiol. Veg.* 13 (2001) 196–223, <http://dx.doi.org/10.1590/S0103-131312001000200008>.
- [45] M. Vágner, Z. Vondráková, Z. Strnadová, J. Eder, I. Macháčková, Endogenous levels of plant growth hormones during early stages of somatic embryogenesis of *Picea abies*, *Adv. Hortic. Sci.* 12 (1998) 11–18.
- [46] P. von Aderkas, M.A. Lelu, P. Label, Plant growth regulator levels during maturation of larch somatic embryos, *Plant Physiol. Biochem.* 39 (2001) 495–502, [http://dx.doi.org/10.1016/S0981-9428\(01\)01271-2](http://dx.doi.org/10.1016/S0981-9428(01)01271-2).
- [47] P. von Aderkas, P. Label, M.-A. Lelu, Charcoal affects early development and hormonal concentrations of somatic embryos of hybrid larch, *Tree Physiol.* 22 (2002) 431–434, <http://dx.doi.org/10.1093/treephys/22.6.431>.
- [48] M. Centeno, R. Rodríguez, B. Berros, A. Rodríguez, Endogenous hormonal content and somatic embryogenic capacity of *Corylus avellana* L. cotyledons, *Plant Cell Rep.* 17 (1997) 139–144, <http://dx.doi.org/10.1007/s002990050367>.
- [49] L. Sáenz, A. Azpeitia, C. Oropeza, L.H. Jones, K. Fuchsova, L. Spichal, M. Strnad, Endogenous cytokinins in *Cocos nucifera* L. in vitro cultures obtained from plumular explants, *Plant Cell Rep.* 29 (2010) 1227–1234, <http://dx.doi.org/10.1007/s00299-010-0906-9>.
- [50] T. Gaspar, C. Kevers, C. Penel, H. Greppin, D.M. Reid, T.A. Thorpe, Plant hormones and plant growth regulators in plant tissue culture, *In Vitro. Cell. Dev. Biol. – Plant.* 32 (1996) 272–289, <http://dx.doi.org/10.1007/BF02822700>.
- [51] L. Hao, L. Zhou, X. Xu, J. Cao, T. Xi, The role of salicylic acid and carrot embryogenic callus extracts in somatic embryogenesis of naked oat (*Avena nuda*), *Plant Cell. Tissue Organ Cult.* 85 (2006) 109–113.
- [52] J.P. Luo, S.T. Jiang, L.J. Pan, Enhanced somatic embryogenesis by salicylic acid of *Astragalus adsurgens* Pall.: Relationship with H<sub>2</sub>O<sub>2</sub> production and H<sub>2</sub>O<sub>2</sub>-metabolizing enzyme activities, *Plant Sci.* 161 (2001) 125–132, [http://dx.doi.org/10.1016/S0168-9452\(01\)00401-0](http://dx.doi.org/10.1016/S0168-9452(01)00401-0).
- [53] F. Quiroz-Figueroa, M. Méndez-Zeel, A. Larqué-Saavedra, V. Loyola-Vargas, Picomolar concentrations of salicylates induce cellular growth and enhance somatic embryogenesis in *Coffea arabica* tissue culture, *Plant Cell Rep.* 20 (2001) 679–684, <http://dx.doi.org/10.1007/s002990100386>.
- [54] F.R. Quiroz-Figueroa, R. Rojas-Herrera, R.M. Galaz-Avalos, V.M. Loyola-Vargas, Embryo production through somatic embryogenesis can be used to study cell differentiation in plants, *Plant Cell. Tissue Organ Cult.* 86 (2006) 285–301, <http://dx.doi.org/10.1007/s11240-006-9139-6>.
- [55] Y. Kanno, Y. Jikumaru, A. Hanada, E. Nambara, S.R. Abrams, Y. Kamiya, M. Seo, Comprehensive hormone profiling in developing Arabidopsis seeds: examination of the site of ABA biosynthesis, ABA transport and hormone interactions, *Plant Cell Physiol.* 51 (2010) 1988–2001, <http://dx.doi.org/10.1093/pcp/pcq158>.