UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
FACULDADE DE FARMÁCIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

Metabolismo de alcaloides e flavonoides em culturas de *Rhodophiala bifida* (Amaryllidaceae) e *Trifolium pratense* (Fabaceae)

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PORTO ALEGRE, 2017

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Metabolismo de alcaloides e flavonoides em culturas de *Rhodophiala bifida* (Amaryllidaceae) e *Trifolium pratense* (Fabaceae)

Tese apresentada por **Andressa Reis** para obtenção do GRAU DE DOUTOR em Ciências Farmacêuticas

Orientador: Prof. Dr. José Angelo Silveira Zuanazzi

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“Um cientista no laboratório não é um mero técnico: é uma criança confrontando fenômenos naturais que são tão impressionantes quanto contos de fada. “

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Resumo

A família Amaryllidaceae apresenta variedades ornamentais com alto interesse comercial e um grupo de compostos chamados Alcaloides de Amaryllidaceae, que apresentam comprovadas atividades biológicas. Devido à baixa produção de compostos relacionados ao metabolismo secundário, são procuradas alternativas para o aumento da produção pois opções como a síntese química total se tornam bastante onerosas. Sendo que uma grande parte dos alcaloides produzidos por essas plantas é obtida por meio de extratos com plantas cultivadas, as culturas de tecido *in vitro* surgem como uma opção interessante. A Fabaceae é a terceira maior família de plantas, com grande diversidade na produção de metabolitos secundários. As investigações fitoquímicas revelaram que esta família é composta por plantas com metabólitos secundários chamados de isoflavonas, compostos fenólicos conhecidos por suas atividades fitoestrógenas. Dessa forma, buscando estudar plantas de ambas as famílias, optou-se pela utilização de *Rhodophiala bifida*, com grande biossíntese da molécula montanina e *Trifolium pratense*, que também é conhecido pelos altos níveis de isoflavonas. Assim, nas plantas de *R. bifida*, buscou-se promover a regeneração dos bulbos, realizar as análises fitoquímicas quali e quantitativas, buscar sequencias relativas a dois genes relacionados à biossíntese da montanina, analisar a expressão destes genes nos diferentes tecidos e tipos de cultivo nos quais cresceram estas plantas e determinar a melhor forma de cultivo dentre as avaliadas. Para as plantas de *T. pratense*, o objetivo foi introduzir as plantas no cultivo *in vitro*, produzir culturas celulares e de raízes, realizar as análises fitoquímicas qualitativas e quantitativas do conteúdo presente nas culturas e promover a elicitação das diferentes linhagens de culturas de raízes, determinar a melhor forma de cultivo dentre as estudadas. Assim, realizou-se a coleta de bulbos de *R. bifida* em três anos consecutivos e posteriormente, foi estabelecido o protocolo para cultivo *in vitro*, com os bulbos de uma destas coletas, a regeneração ocorreu via organogênese direta, com a multiplicação e crescimento das plantas, elas passaram por aclimatização e o cultivo em casa de vegetação, assim como a reintrodução em solo dos bulbos coletados, foi também desenvolvido protocolo para germinação de sementes de *R. bifida in vitro*. Com estas amostras, realizou-se a quantificação de montanina e a identificação dos

alcaloides presentes nas plantas selvagens, da mesma forma, promoveu-se a identificação e caracterização de dois genes importantes da rota biossintética das Amaryllidaceae, o 4OMT e CYP96. Ao final, os maiores incrementos da produção de montanina foram encontrados em plantas selvagens cultivadas em casa de vegetação, com condições controladas, e a identificação das moléculas presentes nas plantas de *R. bifida*, assim como os genes da rota de biossíntese destas plantas, são importantes informações para o aperfeiçoamento de estudos futuros com as mesmas. Nos estudos relacionados ao *T. pratense*, coletou-se as plantas selvagens e as sementes, as quais, foram germinadas *in vitro*, cultivadas e a partir das plântulas, desenvolveu-se protocolos de culturas celulares e culturas de raízes transformadas com *A. rhizogenes*. Com estas amostras foram realizadas as análises quantitativas das isoflavonas principais, daidzeína, genisteína, formononetina e biochanina A, assim como o conteúdo total destas. As amostras de culturas de raízes tiveram seus isoflavonóides identificados e foram elicitadas com sacarose e ácido salicílico para a visualização dos incrementos ou diminuições dos conteúdos ao se utilizar estas moléculas. Por fim, concluiu-se que dentre todos os modos de cultivo avaliados, nas culturas celulares detectou-se menores quantidades de isoflavonas enquanto os melhores resultados foram encontrados nas culturas de raízes quando elicitadas com sacarose 60 g L^{-1}. Considerando-se em conjunto, os resultados encontrados em todo o estudo contribuem para o desenvolvimento de estudos visando a preservação destas plantas, promovendo sistemas de produção *in vitro* ou em casa de vegetação, com maior rendimento das moléculas de interesse.

Palavras-chaves: Montanina, isoflavonas, trevo vermelho, raízes em cabeleira, culturas celulares, N4OMT, CYP96.

Abstract

The Amaryllidaceae family has ornamental varieties of high commercial interest and a group of compounds called Amaryllidaceae alkaloids, which have proven biological activities. Due to the low production of compounds related to secondary metabolism, alternatives are sought for the increase of the production because options like the total chemical synthesis become quite onerous. Since a large part of the alkaloids produced by these plants is obtained by means of extracts with cultivated plants, *in vitro* tissue cultures appear as an interesting option. Fabaceae is the third largest family of plants, with great diversity in the production of secondary metabolites. Phytochemical investigations revealed that this family is composed of plants with secondary metabolites called isoflavones, phenolic compounds known for their phytoestrogenic activities. In order to study plants of both families, *Rhodophiala bifida*, which produces montanine and *Trifolium pratense*, which is also known for yielding high levels of isoflavones. Thus, in the plants of *R. bifida*, we tried to promote the regeneration of bulbs, to carry out the qualitative and quantitative photochemical analyzes, to look for the sequences of two genes related to the biosynthesis of montanine, to analyze the expression of these genes in different tissues and types of cultivation in which these plants were grown and to determine the best form of cultivation. For *T. pratense* plants, the objective was to introduce the plants to *in vitro* cultivation, to produce cell and hairy root cultures, to carry out the qualitative and quantitative phytochemical analysis of the said cultures to promote elicitation of the different hairy root lineages and determine the best form of cultivation. Thus, the harvest of *R. bifida* bulbs in field was carried out in three consecutive years and the protocol for *in vitro* cultivation was established with the bulbs of one of these harvests. Regeneration occurred via direct organogenesis, with the multiplication and growth of the plants which underwent acclimatization and greenhouse cultivation followed by reintroduction of the bulbs in soil, a protocol was also developed for germination of *R. bifida* seeds *in vitro*. With these samples, the quantification of montanine, as well as identification of the alkaloids present in the wild plants were carried out the identification and characterization of two important genes of the biosynthetic route of Amaryllidaceae, 4OMT and CYP96, was done. Largest

increases in montanine production were found in wild plants grown under greenhouse conditions. The identification of the molecules presents in *R. bifida* plants, as well as some of the genes of the biosynthesis route of these metabolites constitute important information for future studies with this important class of plants. In the studies related to *T. pratense*, wild plants were harvested, they were germinated *in vitro*, grown and from the seedlings protocols of cell cultures and roots cultures transformed with *A. rhizogenes* were developed. With these samples, the quantitative analyzes of the main isoflavones, daidzein, genistein, formononetina and biochanin A, as well as their total content were carried out. Root culture samples had their isoflavonoids identified and were elicited with sucrose and salicylic acid. It was concluded that in all the evaluated culture modes, cell cultures had smaller amounts of isoflavones and the best yeilds were found in root cultures when elicited with sucrose 60 g L^{-1}. Taken together, results found throughout this research contribute to the development of protocols at the preservation of these plants, promoting alternatives to their production *in vitro* or in greenhouse, providing systems for increased supply of the molecules of interest.

Keywords: Montanine, isoflavones, red clover, hairy roots, cell cultures, N4OMT, CYP96.

Sumário

Plantas e produtos naturais representam uma ampla fonte de compostos com importantes estruturas químicas e atividades medicinais, podendo fornecer produtos químicos naturais valiosos que servem de ponto de partida para o desenvolvimento de novos fármacos (GURIB-FAKIM, 2006; ZUPKÓ *et al*., 2009).

Em geral, uma planta herbácea leva cerca de dois anos, desde germinação das sementes até a formação das flores, podendo ser propagada ou sofrer divisão bulbar. O processo de obtenção das moléculas de origem vegetal seria de fácil realização por meio de síntese ou semi-síntese química, entretanto devido à complexidade química dos extratos e dos produtos que os compõem, esta estratégia muitas vezes apresenta alto custo devido ao baixo rendimento (45-55%), e dessa forma, as moléculas são mais eficientemente adquiridas por meio da própria planta (EICHHORN *et al*., 1998; HUANG; KUTCHAN, 2000; SALIBA; PTAK; LAURAIN-MATTAR, *2015*; WU; WANG; SIMON, 2003). As moléculas do metabolismo secundário apresentam um volume de produção mundial bastante limitado e as quantidades obtidas a partir da extração são, em comparação com as do metabolismo primário, muito pequenas, elevando o valor de mercado de moléculas com potencial farmacológico, estimado na ordem de várias centenas de milhões de dólares (HUANG; KUTCHAN, 2000).

Fabaceae é a terceira maior família de plantas, com grande diversidade na produção de metabolitos secundários, sendo caracterizada pela presença de isoflavonas, cumarinas, antraquinonas, glicosídeos cianogênicos, glucosinolatos, terpenoides e alcaloides (WINK, 2013). Algumas subfamílias contêm moléculas restritas, como é o caso das isoflavonas, uma subclasse de compostos fenólicos conhecidos como fitoestrógenos (KOWALSKA *et al*., 2014).

A atividade destas moléculas se baseia na similaridade estrutural com o estrogênio 17β-estradiol, Fig. 1***(5)***, e podem atuar como agonistas ou antagonistas, com ação dose-dependente (WU; WANG; SIMON, 2003). Estudos clínicos têm demonstrado atividades no alívio dos sintomas da menopausa, prevenção da osteoporose, hipertrofia benigna prostática, terapia de reposição hormonal, doenças cardiovasculares, hipertensão e tumores hormônio-dependentes (BECK; ROHR; JUNGBAUER, 2005; CEOLATO; SCHAPOVAL; AGNOL, 2012; ERCETIN *et*

al.2012; HEINONEN; WAHALA; ADLERCREUTZ, 2002; KOWALSKA *et al*., 2014; NISSAN *et al*., 2007; SPAGNUOLO *et al*., 2014; WUTTKE *et al*., 2006; XU *et al*., 2015).

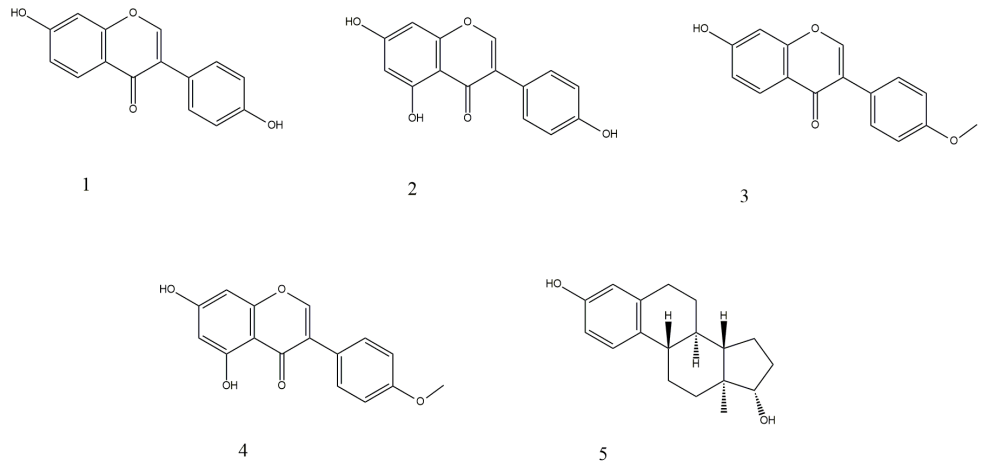

Figura 1. Isoflavonas: daidzeína (**1** - ChemSpider ID 4445025), genisteína (**2** - ChemSpider ID 4444448), formononetina (**3** - ChemSpider ID 4444070) e biochanina A (**4** - ChemSpider ID 4444068) e a molécula de estrogênio 17β-estradiol (**5** - ChemSpider ID 5554).

As isoflavonas são moléculas ocorrem em abundância em espécies de Leguminosas, em plantas como o trevo-vermelho (*Trifolium pratense* L.), conhecido pela presença de daidzeína, Fig. 1 ***(1)***; formononetina, Fig. 1 ***(3)***; biochanina A, Fig. 1 ***(4)***, e genisteína, Fig. 1 ***(2)***, esta última apresentando a mais alta capacidade estrogênica, com grande afinidade de ligação com os respectivos receptores (KOWALSKA *et al*., 2014; SAVIRANTA *et al*., 2010).

Os usos de extratos de plantas selvagens de trevo-vermelho têm muitas vezes limitações por apresentarem grande variabilidade no conteúdo de isoflavonas na sua composição química. O principal problema enfrentado nos testes de atividade com esses extratos seria a falta de uma padronização química dos conteúdos desses fitoestrógenos (SPAGNUOLO *et al*., 2014). Tais flutuações seriam devidas a variações sazonais, produção em estádios específicos de desenvolvimento, estresse, disponibilidade de nutrientes e condições do solo. Dessa forma, muito tem sido estudado com relação à possibilidade de produção *in vitro* destas plantas como uma alternativa comercial na obtenção destes metabólitos de forma mais padronizada (NISSAN *et al*., 2007; VERPOORTE; CONTIN; MEMELINK, 2002).

As culturas celulares consistem em células livres ou em pequenos grupos provenientes de calos friáveis e cultivadas em meio líquido, apresentam um crescimento relativamente rápido e são normalmente homogêneas. Com a ativação de caminhos metabólicos adequados, os produtos secundários podem por vezes se acumular em níveis mais elevados em células do que em plantas inteiras e ser usados, dessa forma, para fins medicinais (YUE *et al*., 2014).

Como alternativa à cultura celular, a tecnologia de cultura de raízes em cabeleira (*hairy roots*) pode ser utilizada. Estas culturas podem ser produzidas usando cepas de *Agrobacterium rhizogenes* e serem mantidas por longos períodos, podendo ser utilizadas para produção em larga escala por apresentarem estabilidade genética e crescimento sem a necessidade da adição de suplementos hormonais (DIOP *et al*., 2007).

Visando um incremento na produção dos metabólitos secundários, pode-se utilizar estímulos de origem biótica ou abiótica, chamados de elicitores, que reorientarão a biossíntese de moléculas do metabolismo primário e secundário (WILSON; ROBERTS, 2014). O uso de elicitores foi identificado como um estimulador da biogênese de estruturas como terpenos, flavonoides, alcaloides e fenilpropanoides (ZHAO; DAVIS; VERPOORTE, 2005).

As espécies de Amaryllidaceae são conhecidas por suas variedades ornamentais e a presença de um grupo exclusivo de metabólitos, os alcaloides de Amaryllidaceae (GABRIELSEN *et al*, 1992; BASTIDA *et al*, 2011). Estes metabólitos têm sido amplamente estudados para atividades biológicas relacionadas à inibição da acetilcolinesterase, com utilização no tratamento da doença de Alzheimer e da demência por meio do alcaloide galantamina, assim como no combate ao melanoma, câncer cerebral e glioblastoma testados com licorina, haemantamina, tazetina e narciclasina (TAKOS; ROOK, 2013).

Rhodophiala bifida (Herb.) Traub é uma Amaryllidaceae que apresenta grandes concentrações do alcaloide montanina (Fig. 2), este alcaloide apresenta atividades relacionadas a ações antimicrobianas (CASTILHOS *et al*., 2007), citotóxica *in vitro* para diferente células de câncer (PAGLIOSA *et al*., 2010), ansiolítica, antidepressiva, anticonvulsivante e anti-inflamatória (FARINON *et al*., 2015; SILVA *et al*., 2006).

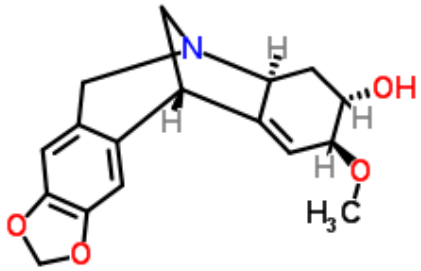

Figura 2. Molécula do alcaloide montanina (ChemSpider ID 9263081).

Com o crescente interesse relacionado à produção de plantas medicinais, surgem estudos com relação à regeneração destas plantas *in vitro*, buscando a produção de variedades com alta qualidade e incremento na formação de moléculas alvo. Estes tipos de cultivo conseguem manter a capacidade de síntese de alcaloides idênticas as da planta selvagem ou ainda possibilitam a formação de outras variedades com características desejáveis (AHMAD *et al.*, 2013). Há possibilidade de aumento da produção de moléculas desejadas por manipulação das condições ambientais que são fortemente ligadas ao metabolismo secundário da planta ou dos seus tecidos em cultivo, e os reguladores de crescimento têm sido utilizados com sucesso como elicitores na indução de alcaloides (BERGONON *et al.*, 1996; GEORGIEV *et al.*, 2011; IVANOV *et al.*, 2011).

Devido ao fato de os alcaloides de Amaryllidaceae serem produzidos em pequenas quantidades nas plantas, frente à demanda comercial, o desenvolvimento de rotas de síntese razoavelmente curtas e que possam ser utilizadas para a produção de compostos em uma escala comercial e economicamente viável seria importante. Porém, a via biossintética para a produção da maioria destas moléculas permanece pouco conhecida e algumas enzimas e intermediários metabólicos aguardam descoberta, tornando-se um ponto limitante no uso biotecnológico desta família (SINGH; DESGAGNÉ-PENIX, 2014).

Objetivos:

Com base no exposto, devido à importância medicinal das moléculas produzidas nestas duas famílias de plantas, em especial *Rhodophiala bifida* e *Trifolium pratense*. Os objetivos gerais deste trabalho foram os seguintes:

I) Para *R. bifida*

- Promover a regeneração dos bulbos, realizar as análises fitoquímicas quali e quantitativas, buscar as sequências relativas a dois genes relacionados à biossíntese da montanina, analisar a expressão destes genes nos diferentes tecidos e tipos de cultivo nos quais cresceram estas plantas, determinar a melhor forma de cultivo dentre as avaliadas.

II) Para *T. pratense*

- Introduzir as plantas no cultivo *in vitro*, produzir culturas celulares e de raízes, realizar as análises fitoquímicas qualitativas e quantitativas do conteúdo presente nas culturas e promover a elicitação das diferentes linhagens de culturas de raízes, determinar a melhor forma de cultivo dentre as estudadas.

Devido à organização dos assuntos e experimentos, este trabalho será apresentado na forma de artigos. Os Materiais e Métodos estarão contemplados em cada artigo de acordo com o desenvolvimento do mesmo.

CAPÍTULO I

Production of Amaryllidaceae alkaloids by *in vitro* plant systems: a biochemical approach

(Review)

Introduction

Natural products have an important role in treatment and prevention of human diseases. Studies conducted from 1981 to 2002 reported the identification of natural products representing around 28% of the new chemical entities used as commercialized medicines. An additional 24% of the drugs are synthesized or semi-synthesized based on natural products chemistry, resulting in more than 50% of marketed drugs derived from natural sources or serving as inspiration for new drugs (Chin *et al*., 2006). Using these natural products as drugs or models to design new ones greatly enhances the chances of success in the development of promising new medicines (Chin *et al*., 2006) when pharmacological activity was already described.

Alkaloids represent a highly diverse group of small molecular weight compounds related to each other by the presence of a nitrogen atom in a heterocyclic ring. Most of these compounds are derived from amines, produced by the decarboxylation of amino acids, such as histidine, lysine, ornithine, tryptophan and tyrosine (Nakagawa *et al*., 2011). Alkaloids are present mainly in the plant kingdom and in less amount in microorganisms, vertebrates and invertebrates. Currently, drugs containing alkaloids as active principle are vastly commercialized, and generally obtained or inspired from plants producing alkaloids such as morphine and codein (analgesic), vinblastine (anticancer), sanguinarin (antibiotic), scopolamine (sedative) and galanthamine (acetylcholinesterase inhibitor). Other more common plant alkaloids include caffeine nicotine, cocaine, theophylline and synthetic acetylated morphine (Facchini, 2001; Hashimoto and Yamada, 1994; Ziegler and Facchini, 2008).

Alkaloids can be categorized according to their amino acid precursor. Amaryllidaceae Alkaloids (AmAl) constitutes an exclusive group of alkaloids, derived from tyrosine. The first AmAl isolation is reported from 1877, and until now includes more than 500 diverse compounds representing a complex alkaloid. These AmAl are exclusive from Amaryllidaceae plants and comprise twelve different chemical skeletons corresponding to their ring organizations (Dewick, 2009; Guo, 2015).

The most representative molecules of this family present numerous biological activities as described in Table 1. For example, the well-known galanthamine **(37)** alkaloid is a reversible and competitive inhibitor of the acetylcholinesterase as well as an allosteric modulator of the nicotinic receptor for acetylcholine. In the form of hydrobromide salt, known as Razadyne® or Razadyne ER®, it is one of the most important commercial products that can reduce the process of neurological degeneration in Alzheimer's disease (AD). This compound presents long- acting, specific activity, reduced side effects, and can also be useful in the treatment of other forms of dementia than AD and poliomyelitis (Bastida *et al*., 2006; De Andrade *et al*., 2012; Heinrich and Teoh, 2004; Maelicke *et al*., 2001; Thomsen *et al*., 1990).

Due to the wide range of biological activities of these molecules, or because of the economic value of the galanthamine, the number of studies concerning this family of plants has grown in the past decade. Owing to the demands of the world market, the search for new bioactive molecules or different properties in these diverse structural molecules (de Andrade *et al*., 2015; Giordani *et al*., 2011; Guo, 2015; Iannello, 2014; Ortiz *et al*., 2012) resulted in the use of species with slow-growing or slow-reproducing properties, with the risk of extinction of the biological resources (Voeks and Leony,

2004; Zschocke *et al*., 2000). Therefore, alternatives to produce these natural compounds from the plants are explored since the partial and/or total synthesis still require improvement (Diamond and Desgagné-Penix, 2015; Saliba *et al*., 2016). Tissue culture technologies represent alternatives to produce these plant secondary metabolites in high-quality production systems (Ahmad *et al*., 2013).

As previously reported, *in vitro* culture systems were considered as alternatives to produce these types of alkaloids (Berkov *et al*., 2005; Saliba *et al*., 2015). Galanthamine is an example of AmAl produced in plantlets, shoot-clumps and callus cultures. Other AmAl have been supplied using different biotechnology systems or using genetic engineering to enhance the production *in vitro* (Berkov *et al*., 2010).

In this review, we present studies related to *in vitro* production of AmAl in different biotechnological systems and/or organs. We also describe the latest discoveries, developments and improvements in the search to yield these important molecules by *in vitro* tissue culture.

Alkaloids in the Amaryllidaceae family

Amaryllidaceae belongs to the Asparagales order and encompasses monocotyledons, herbaceous and commonly perennial and bulbous flowering plants. This family comprises around seventy-five genus and approximately 1600 species distributed in tropical and subtropical areas of the world (Guo, 2015). Plants from this family are characterized by underground stem and bulbs, rarely rhizomatous. Leaves can be simple and rather fleshy, lanceolate, grouped on stem base or arranged alternately on it. Flowers usually contain three petals and three sepals in a range of colors including red, orange, yellow, pink, white and rarely blue. Fruits are capsule-shaped or berrylike. In their

majority, Amaryllidaceae plants are grown as ornamentals or potted plants, covering genus such as *Amaryllis, Polianthes, Galanthus, Griffinia, Worsleya, Lycoris* and *Narcissus* and this family of plants is in the top 20 most widely considered medicinal plant families (Encyclopædia Britannica, 2014; Jin and Xu, 2013).

The chemical composition of Amaryllidaceae reveals a large amount of pharmacologically active compounds such as phenols, lectins, peptides and alkaloids, representing more than 500 isolated molecules to date (Jin and Xu, 2013). The AmAl represent the most important chemical group among them and are largely restricted to this family, precisely to the subfamily Amaryllidoideae in the genus *Hosta* (Asparagales) (Chase *et al*., 2009). These compounds can be classified into a dozen of distinct chemical scaffolds according to their ring systems, structurally similar to isoquinoline alkaloid group (Berkov *et al*., 2014; Zayed *et al*., 2011).

AmAl biosynthesis formation requires the protocatechuic aldehyde and tyramine precursors. In the first stages of AmAl biosynthesis, Phenylalanine ammonia-lyase (PAL) catalyzes the elimination of ammonia from phenylalanine, generating *trans*-cinnamic acid (Fig.1), and then, an enzyme of the cytochrome P450 superfamily (CYP73A1) converts it to *p*-coumaric acid. This molecule is transformed by another P450 enzyme (CYP98A3) into 4-hydroxycinnamic acid (caffeic acid) or into 4-hydroxybenzaldehyde. Both will then give Protocatechuic aldehyde **(1)**. It is believed that caffeic acid transformation requires the enzymatic action of a V*p*VAN paralogue, an enzyme that was discovered in *Vanilla planifolia*. This protocatechuic aldehyde belongs to the phenylpropanoid pathway. The biosynthesis of the AmAl precursor tyramine is analogous to isoquinoline alkaloids biosynthesis. The tyrosine

decarboxylase (TIDC) necessary for tyramine formation is the only regulatory key enzyme in the production of many alkaloids in plants (Kilgore and Kutchan, 2015; Kornienko and Evidente, 2008; Singh and Desgagné-Penix, 2014).

The structural similarity of the Amaryllidaceae alkaloids could result from the existence of this unique norbelladine **(3)** intermediate formed by the condensation, using an unknown reductase, of protocatechuic aldehyde and tyramine **(2**) (Fig.1). The tetrahydroisoquinoline core (Schiff-base) present in the norbelladine is fundamental for AmAl structural diversity. Norbelladine **(3)** is then methylated by norbelladine 4'-*O*-methyltransferase (N4OMT) to form 4'-*O*-methylnorbelladine **(4)** which serves as a key intermediate for multiple biosynthetic pathways, giving rise to various structural types of AmAl (Kilgore and Kutchan, 2015).

A crucial step in the AmAl biosynthesis is the 4'-*O*-methylnorbelladine cyclisation by an oxidative phenol-phenol coupling enzyme, which can occur in the *ortho-para, para-ortho* or *para-para* positions and will result in the different structural backbones described so far (Fig. 1). This results in a split of the Amaryllidaceae biosynthetic pathway with the para-para' coupling generating the crinine and haemanthamine enantiomeric series, the ortho-para'phenol coupling giving the classical lycorine alkaloid and the para-ortho'phenol coupling, giving galanthamine. All the other alkaloid backbones are derived from these four skeletons (Kilgore and Kutchan, 2015).

Each Amaryllidaceae produces its own groups of alkaloids, some of them being major compounds and some others present in multiple forms at lower concentrations. These variations result from differences in substrate specificity and in levels of expression of various enzymes like *O*- and *N*-methyltransferases (OMTs, NMTs), in C-C and C-O

bond formation and in reactions of oxidation, reduction, demethylation and hydroxylation. As a consequence AmAl production results in a range of diverse structures, differing among species, cultivars and/or tissues, or even from the environments were the plants are living (Singh and Desgagné-Penix, 2014; Takos and Rook, 2013).

The wide variety of molecules, reactions and genes present in the Amaryllidaceae family were not completely discovered yet, although in the last years progresses in this area have been massive. Transcriptomic studies have been initiated with plants like *Lycoris aurea* (Wang *et al*., 2013), *Lycoris radiata* (Jiang *et al*., 2011), *Narcissus* sp. *aff. Pseudonarcissus*, *Galanthus* sp. and *Galanthus elwesii* (Kilgore *et al*., 2014, 2016a) and with this information, putative genes involved in the AmAl biosynthetic pathway, like *LrPAL*, *TYDC OMT*, *NMT*, *N4OMT* were described. The *para-para'* C-C phenol coupling cytochrome *P450* (CYP96T1) is the first enzyme of this type characterized in monocot and it is responsible for the formation of the enantiomeric mixture of noroxomaritidine from 4'-*O*-methylnorbelladine. This enzyme can also catalyze, with less specificity, a *para-ortho*'phenol coupled product, the *N*-demethylnarwedine (Kilgore *et al*., 2016b). Other research with labeled precursor in *Leucojum aestivum* shoot cultures identified the role of this molecule structure in the biosynthetic pathway (El Tahchy *et al*., 2011). However, many reactions still remain undescribed and investigators have only hypothesis about the genes involved in the reactions. Their identification remains thus a significant challenge.

In vitro alkaloids production

Most secondary metabolic products can be obtained directly from plant extraction. However, it sometimes requires large harvest to obtain low amounts of these crude extracts and consequently, few milligrams of isolated alkaloid. Furthermore, many plant species used to obtain those extracts, are slow growing and/or difficult to grow in open field. Consequently, harvesting can occasionally cause depletion of wild species and lead to extinction. The climate and soil conditions that can influence the quality and the quantity of secondary metabolites might be season dependent, related to the circadian rhythm, to the development, temperature, water availability, ultraviolet radiation, nutrients (macro and micronutrients) availability, altitude, pollution, induced by mechanical stimuli or by pathogen attack (Gobbo-neto and Lopes, 2007; Miralpeix *et al*., 2013; Namdeo, 2007; Saliba *et al*., 2015). Secondary metabolite extraction can thus be variable when done on plants grown in natural conditions.

Chemical synthesis of natural products can be an alternative to obtain chemicals without harvesting, but very often low yields are obtained or the synthesis is economically impractical due the structural complexity of these compounds (Giri and Zaheer, 2016; Samanani *et al*., 2004).

Large-scale production of secondary metabolites has been widely explored. Studies involving plant biotechnology provide opportunities to explore production by microorganisms, cells, tissues, organs and whole organisms *in vitro*. These studies aimed at the production of the desired compound naturally and/or by performing genetic manipulations. Plant or organ (leaves, shoots, meristems, roots or embryos) cultivation can be established *in vitro.* All the conditions for such procedures are sterile and

appropriated culture media for plant material development, proliferation and mimicking the chemical environment of wild plants are used (Giri and Zaheer, 2016).

Cultures of embryo, callus, bulbs, shoots, cell suspensions and intact plant from Amaryllidaceae species were the subject of many researches in the last four decades (Table 2), some including protocols for initiating *in vitro* cultures and for micropropagation. Since the Amaryllidaceae for ornamental flowers with economic interest, most of these studies aimed at plant improvement. Normally, this process is long but still shorter than field production and allows producing great quantity of plants with relatively low investments.

Alkaloid production using *in vitro* grown plants can also be affected by many factors. A review of the studies listed in the Table 3 shows that the salt based medium mostly used for these experiments is MS medium (Supplementary material – Table SM1) in 79% of the cases, followed by 1/2 MS in 12% of the studies, and by other less frequently used, such as G media, Gamborg, MMS, N6 and NL. The quantity of sucrose (Supplementary material – Table SM2), was analyzed as well, and 30 g L^{-1} was employed in 41% of the investigations, 60 g L^{-1} in 32% of them, followed by 90 g L^{-1} (18%) and 20 g L^{-1} used in less than 10% of the scientific studies with Amaryllidaceae plants.

With regard to plant growth regulators (GR, Supplementary material – Table SM3) used to maintain or produce the explants or the Amaryllidaceae cultures, BA is the most frequently used GR, at 1 and 2 mg L^{-1}. The auxin NAA is used at 0.1; 0.5 and 1 mg L^{-1}, follow by other auxin used in large quantities, 2,4-D at 1 mg L^{-1}. Table SM3, compare the utilization of these GR, their most used concentrations in Amaryllidaceae family tissue culture and the number of citations in the literature consulted for this review.

It is also known that several factors and practices can affect the synthesis of the molecules inside of the plants. These can be changed to increase the generation of alkaloids in order to maximize their production for commercial purpose and exploitation of these metabolites (Ahmad *et al*., 2013). The studies listed in Table 3 and in the tables of the supplementary material indicate that for maximum yield, the MS medium (Table SM4) was used in 97% of the times, (Table SM5) liquid medium in 55 % of them, the sucrose at 30 g L^{-1} (Table SM6) in 72 % of the studies and shoot clumps cultures (Table SM7) in 67%. Similarly, alkaloids production might be affected by the use of different growth regulators (Table SM8). BA (2 mg L^{-1}) was used in 13% of the studies, NAA 1.2 mg L^{-1} was the second most applied, followed by NAA 1.9 mg L^{-1} (8%), 2,4-D 1 mg L^{-1}, BA 0.1 and 5 mg L^{-1} in 6,4% of the studies. Other GR less described in the literature are included in the Table SM8, together with the type of alkaloids produced *in vitro*.

Few studies (Table 3) also make use of bioreactors and systems such as the temporary immersion (TIS) in RITA® (*Récipient à Immersion Temporaire Automatique*). Some of them showed an increase biomass production as well as a gain in alkaloids production. These studies (Table SM9) used liquid medium with cultures cultivated in orbital shaker at 110 rpm in 28% of the reports, the temporary immersion system in 39 % of the times, with 22% of them in Rita ® Bioreactors. Other studies use shakers at different rotations speeds or without movement, or even in bubble column bioreactors.

Metabolic engineering in the Amaryllidaceae alkaloids production

Metabolic engineering is a tool that enables to improve the synthesis of specific compounds in cells and organisms by modulating genes coding for key enzymes, protein transport or regulators, or acting as limiting factor of biosynthetic pathways. Plants from

Amaryllidaceae family, while extensively studied for phytochemistry and pharmacology, are still underexplored in terms of ecology, physiology and molecular studies, and most of the genes for AmAl biosynthesis remain unknown (Takos and Rook, 2013).

Techniques for production of AmAl by metabolic engineering in heterologous systems include microorganisms, cell culture or plant organs and intact plants. In contrast to the chemical synthesis, biological systems have the theoretical advantage to produce large amount of specific desired compounds with the convenience of causing lower environmental damages at reduced costs (Glenn *et al*., 2013).

Genetic modification of ornamental geophytes, essentially of the monocotyledonous type is generally impaired by the incapacity to achieve *Agrobacterium* transformation as well as efficient regeneration (Koetle *et al*., 2015). Some factors which can influence *Agrobacterium* infection and the subsequent steps related to the transformation in these plants are related to plant genotypes, explant types, super-virulent strains, efficient promoters, agrobacterial monolayer, sonication (Trick and Finer, 1997) and may represent the key points to achieve the transformation of these plants in the future (Cheng *et al*., 2004; Koetle *et al*., 2015).

Due to all these points, there is a reduced number of studies related to genetic transformation with Amaryllidaceae plants. The research in this field started in 1984 (Table 4) with the work of Hooykaas-Van Slogteren and coworkers. They tried in this study to infect *Narcissus* cv. Paperwhite with *A. tumefaciens*. The next paper was published 18 years later (2002), with a different species of *Narcissus*. Basically, the studies of metabolic engineering with Amaryllidaceae plants belong to *Narcissus*,

Hippeastrum and Leucojum genera, using both species of *Agrobacterium* (*A. tumefaciens* and *A. rhizogenes*) to obtain the transformants.

With regard to the future in this field, recent studies have been developed for identifying and studying the genes of the AmAl metabolic pathway (Kilgore *et al*., 2014, 2016a, 2016b; Kilgore and Kutchan, 2015). Others aimed at developing alkaloid metabolomics (Berkov *et al*., 2011; Torras-Claveria *et al*., 2010, 2014) in order to facilitate the discovery of the enzymes and genes of these pathways. These studies will help further elucidating the many questions concerning the biosynthesis process of these alkaloids. Thus, in the not too distant future, the transformation and search for species with increased AmAl production will become feasible, making these compounds economically accessible for drug discovery.

Conclusion and perspective in the future

Amaryllidaceae plants are known as ornamental species and through the economically important molecule, galanthamine. However, this plant family produces molecules specific of different species with an infinity of biological activities to be discovered.

Many of these ornamental plants were introduced *in vitro*, with the aim to be produced more rapidly at reduced costs. These plants were later used to produce alkaloids. Culture conditions were adjusted to increase the production of these compounds as compared to wild plants. All these studies suggest that *in vitro* cultures presenting a certain degree of tissue differentiation produce higher amount of Amaryllidaceae alkaloids.

Metabolic engineering for these species is still in its infancy as compared to other plant families. Recent studies are unraveling the metabolic pathways and enabling us to make

calculated genetic modifications and thereby obtain transformed Amaryllidaceae plants, microorganisms or other more easily grown plants with increased production of the desired molecules on an industrial scale.

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Figures

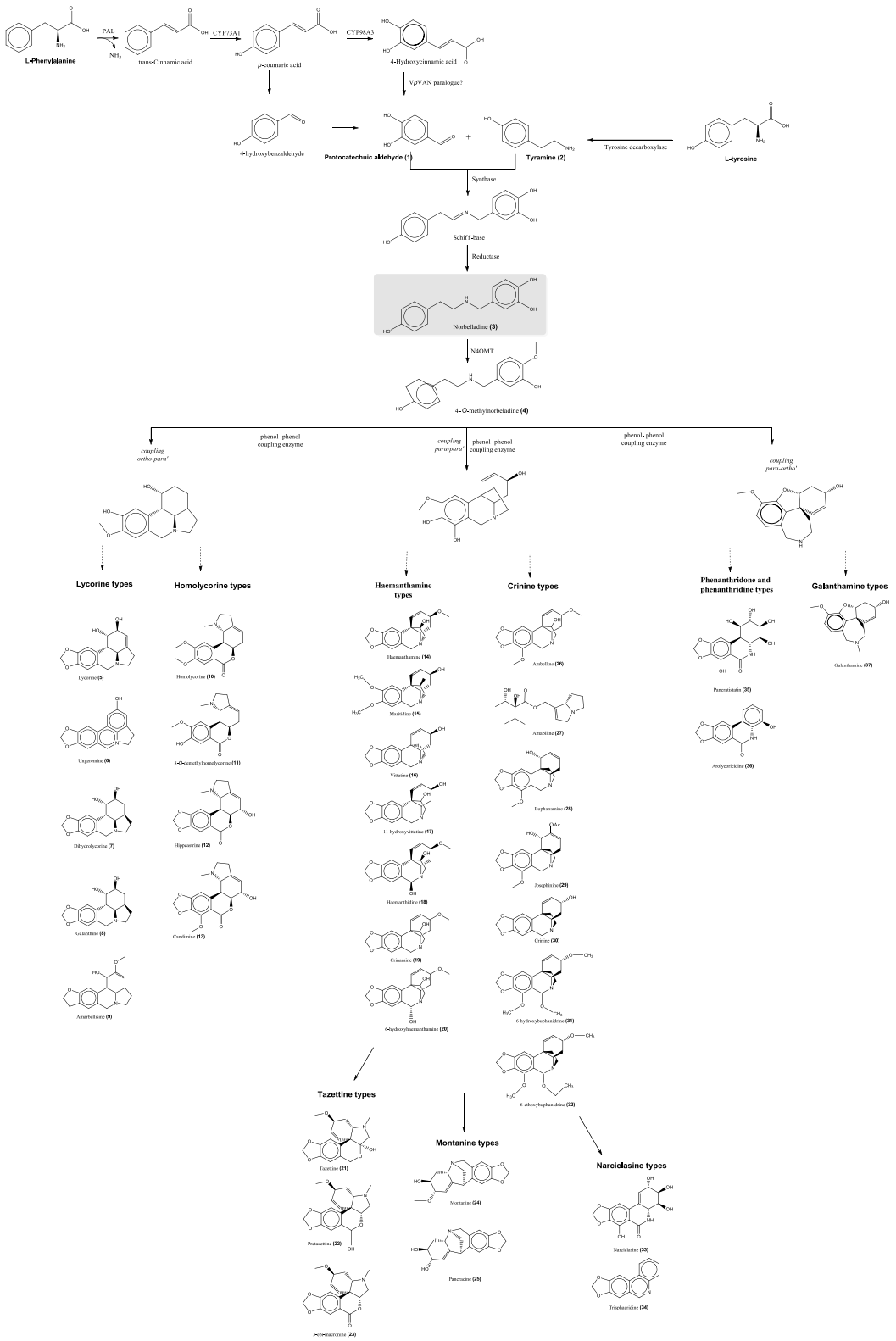

Figure 1 Proposed biosynthetic pathway for the Amaryllidaceae alkaloids showing the main types of skeletons and their core formation (Kilgore and Kutchan 2015).

Tables

Table 1. Biological activities related with the Amaryllidaceae Alkaloids tested over the decades.

Biological activities	Molecule with positive results	Reference
Anxiolytic-, antidepressant- and anticonvulsive effects	Montanine, Figure 1**(24)**.	(da Silva *et al*. 2006)
AchE inhibition	Ungeremine **(6)**, lycorine **(5)** and montanine.	(Rhee *et al*. 2004; Pagliosa *et al*. 2010; De Andrade *et al*. 2012)
Analgesic	Galanthine **(8)**.	(Bastida *et al*. 2006)
Antimicrobial activity	Amarbellisine **(9)**, montanine, vittatine **(16)**, 11-hydroxyvittatine **(17)**, pancracine **(25)**.	(Castilhos *et al*. 2007; Kornienko and Evidente 2008)
Anticancer activity	Tazettine **(21)**.	(Bastida *et al*. 2006)
Antifungal activity	Amarbellisine, hippeastrine **(12)** and lycorine.	(Kornienko and Evidente 2008)
Anti-inflammatory activity	Lycorine and haemanthidine **(18)**.	(Çitoglu *et al*. 1998)
Antimalarial activity	6-Hydroxyhaemanthamine **(20)**, haemanthamine **(14)** and lycorine.	(Sener *et al*. 2003)
Antiprotozoan actions (*Trichomonas vaginalis)*	Candimine **(13)** and lycorine.	(Giordani *et al*. 2010; Giordani *et al*. 2011)
Antiprotozoan actions (*Trypanosoma brucei rhodesiense)*	Galanthine, dihydrolycorine **(7)**, haemanthamine, arolycoricidine **(36)**, haemanthidine.	(Herrera *et al*. 2001; Kaya *et al*. 2011)
Antiprotozoan actions (*T. brucei rhodesiense, Trypanosoma cruzi* and *Plasmodium falciparum)*	Pancracine.	(Bastida *et al*. 2006)
Antiproliferative effects	Amarbellisine, crinine **(30)**, buphanamine **(28)**, 6-hydroxybuphanidrine **(31)**, 6-	(Silva *et al*. 2008; Evidente *et al*. 2009; Berkov *et al*. 2011)

	ethoxybuphanidrine **(32)**, montanine, vittatine.	
Antiretroviral	Lycorine, homolycorine **(10)**, haemanthamine, trisphaeridine **(34)**.	(Bastida *et al*. 2006; Reyes-Chilpa *et al*. 2011)
Antitumor and antimitotic	Lycorine, homolycorine, narciclasine **(33)** and pancratistatin **(35).**	(Bastida *et al*. 2006; Lamoral-Theys *et al*. 2009)
Antiviral	Lycorine, tazettine and hippeastrine.	(Renard-Nozaki *et al*. 1989; Bastida *et al*. 2006)
Apoptosis induction in tumor cells.	Haemanthamine, narciclasine, crinine, lycorine, crinamine **(19)**, ambelline **(26)**, amabiline **(27)**, josephinine **(29)**.	(McNulty *et al*. 2009)
Cytostatic	Lycorine.	(Bastida *et al*. 2006; De Andrade *et al*. 2012)
Cytotoxic	Homolycorine, 8-*O*-demethylhomolycorine **(11)**, hippeastrine, vittatine, maritidine **(15)**, pretazettine **(22)**, 3-epi-macronine **(23)**, tazettine.	(Bastida *et al*. 2006; De Andrade *et al*. 2012)
Hypotensive activity	Galanthine, homolycorine, 8-*O*-demethylhomolycorine, haemanthamine.	(Bastida *et al*. 2006; Kaya *et al*. 2011)
Neurological degeneration in Alzheimer's disease, other types of dementia and poliomyelitis	Galanthamine **(37).**	(Thomsen *et al*. 1990; Maelicke *et al*. 2001; Heinrich and Teoh 2004; Bastida *et al*. 2006; De Andrade *et al*. 2012)

Table 2. Amaryllidaceae plants cultivated by different biotechnology techniques and purposes, during the last four decades.

Species	Main goal of the study	Culture conditions	Reference
Hippeastrum spp. hybrids	Micropropagation protocol for tissue culture.	The explants were inverted scapes and peduncles, cultivated in MS medium with some modifications in macro- ($CaCl_2$, $MgSO_4$, without KH_2PO_4 and with NaH_2PO_4) and micro-nutrients ($MnSO_4$) supplemented with vitamins (nicotinic acid, thiamine and pyridoxine at 1 mg L^{-1}). The medium also contain 2.4-D and BA at 1 mg L^{-1}. Friable calli were obtained from ovary tissue cultured on a medium with 1-naphthaleneacetic acid (NAA) 2mg L^{-1} and BA 4 mg L^{-1}, and produce shoots after 8 weeks. One mother bulb can produce 45 explants that at the end of 8 weeks of culture can produce 450 rudimentary plantlets.	(Seabrook and Cumming 1977)
Narcissus tazetta var. Chinensis Roem	Protocol for *in vitro* culture aiming at increasing the rate of propagation of the bulbs.	The medium used was N6 (Chu and Shannon 1975) supplemented with BA 2 mg L^{-1} and 2.4-D 0.1 mg L^{-1}. The explants were incubated for 3-4 weeks to generate the bulblets.	(Zhen-Guang 1982)
Lilium davidii var. Unicolor Coton	Organogenesis *in vitro*.	MS medium supplemented with NAA 0.2 mg mL^{-1} and BA 2 mg mL^{-1}, no light necessary to promote the induction.	(Pifang *et al*. 1985)
Narcissus tazetta	Callus induction from bulb scales.	MS medium containing BA 1 mg L^{-1} and 2.4-D 0.1 mg L^{-1} was used to induce white compact callus. The transfer to a medium without growth regulators (GR) or using BA 1 mg L^{-1} or NAA 0.1-0.5 mg L^{-1} promoted the growth of adventitious shoots. Most of the bulblets developed leaves and roots when cultured on a ½ MS media with NAA 0.03 mg L^{-1} or without GR.	(Hengsen and Cuihua 1987)
Hippeastrum hybridum	Single and twin scaling and *in vitro* multiplication.	Protocorm-like bodies were regenerated from single scales that finally end up regenerating bulblets. Bulblets directly originate from the twin scales. The medium suggested for multiplication of protocorms was liquid MS medium with zeatin 1.0 mg L^{-1} and for bulblet/shoot formation the indole-3-acetic acid (IAA) 1.0 mg 1 L^{-1} was added to this medium.	(Huang *et al*. 1990)

Narcissus tazetta var. Chinensis Rome	Micropropagation aiming new varieties of *Narcissus*.	The results showed that the bulbils were induced on twin scales using MS supplemented with BA 0-5 mg L^{-1}, NAA 0-1 mg L^{-1} and activated charcoal (AC). The percentage of induction reached 70%. More bulblets could be obtained through subculture if the bulbils were transplanted in ½ MS medium with indole-3-butyric acid (IBA) 0.1mg L^{-1} in shade-culture.	(Yimin and Guoning 1991)
Amaryllis belladonna	Multiplication by tissue culture techniques.	The micro-plants were generated by twin-scales and immature scapes and the highest multiplication rate was founded when the plants were cultivated in medium with BA 22.2 µM and NAA 0.54 µM with a sucrose concentration between 20 to 30 g L^{-1}.	(Bruyn *et al*. 1992)
Crinum macowanii	Regeneration and growth of bulblets from bulb scale explants.	Shoots were induced on MS medium containing NAA or BA 0 to 20 mg L^{-1} and a modified MS medium (MMS medium) containing ancymidol (A-Rest™) 1.25 mg L^{-1}, NAA and kinetin 0.1 mg L^{-1}. Large bulblets (≥ 5 mm) were trimmed and split in half, and secondary plantlets were regenerated on MMS-medium containing kinetin or MS-medium without GR. Using this medium the bulblets were suitable for splitting within 12-16 weeks and possibility producing 700-1000 bulblets from each mother-bulb within 12 months.	(Slabbert *et al*. 1993)
Narcissus tazetta var. Chinensis	The tissue culture dedifferentiation observed in Electron microscopy (EM).	Differentiation of callus and bulblets was promoted using MS medium with BA 1-8 mg L^{-1} and NAA 0.5 mg L^{-1}.	(Weilian *et al*. 1993)
Narcissus var. St. Keverne and Hawera	Regeneration protocol with leaf explants from shoot cultures.	The medium used to induce bulbil was MS containing 176 mM sucrose for both cultivars; NAA 0.54 µM + IAA 5.4 µM + IBA 5.4 µM for "St Keverne" cultivar and IAA 27 µM for "Hawera" cultivar.	(Staikidou *et al*. 1994)
Nerine × *Mansellii*	Proliferation and regeneration of meristematic clusters in bioreactors.	Inflorescence-derived explants of Nerine were cultured on MS supplemented with (2,4-D) 10 µM and BA 10 µM, sucrose 30 g L^{-1} and Difco bacto-agar 9 g L^{-1} generating callus-like tissue interspersed with nodular tissue. Subculture of nodular tissue was made using ½ MS minerals, full strength MS organics, sucrose 30 g L^{-1}, NAA 0.25 µM, BA 10 µM and paclobutrazol (PAC) 8.7 µM in Erlenmeyer flasks or bubble bioreactors. The cultures were incubated at 25°C on a gyratory shaker at 95-100	(Ziv *et al*. 1994)

		rpm, under 16h photoperiod, giving 30 μmol $m^{-2}s^{-1}$. Growth and proliferation in bioreactors were higher than in shaker flasks. Nerine cultures, when cultivated in semi-continuous culture, showed increased growth (100%) in flasks and (140%) in bioreactors; the presence of PAC in the culture period decreased growth and proliferation. Removal of PAC, induced proembryogenic clusters developement.	
Crinum macowanii (Bak.)	Regeneration protocol from floral stem explants.	The highest number of shoots was regenerated via direct organogenesis from immature floral stems (70-100 mm), using middle or basal parts. Discs were orientated with their proximal ends on the medium. Combinations of kinetin 4.65 μM + IAA 0.57 μM (or NAA 0.54 μM) or even BA 4.44 μM + 2,4-D 0.45 μM regenerated highest numbers of shoots. The shoots were rooted on MS-medium containing sucrose 0.17 M.	(Slabbert. *et al*. 1995)
Gladiolus X grandifloras var. Peter Pears	Protocol for cell suspension cultures from friable embryogenic callus using cormels as explants.	Explants were placed on medium consisting of MS, plus thiamine HCl 1 mg L^{-1}, adenine sulfate 5 mg L^{-1}, NaH_2PO_4 80 mg L^{-1}, casein hydrolysate 1 g L^{-1}, sucrose 30 g L^{-1} and 2,4-D 2 mg L^{-1} and Gelrite 2 gL^{-1} to solidify, pH was adjusted in 5.8 and autoclaved for 20 min (12l °C, 138 kPa) to induce callogenesis. The cultures were kept at 24 ℃ in the dark. Friable calli were isolated from the explant, subcultured three times and 2-3 g of this callus were transferred to liquid medium (250 mL) as described above, in a shaker (120 rpm). Primary embryo development was observed on media with zeatin or with BA 0.25 μM.	(Remotti 1995)
Narcissus bulbocodium	Procedure for *in vitro* production of bulbs from twin-scales explants.	MS medium supplemented with BA 4 mg L^{-1} + NAA 0.12 mg L^{-1} + IBA 1 mg L^{-1} promoted the formation of shoots and leaves. The use of BA + IBA induces the growth of tiny bulbs. Bulb growth required sucrose at 9%.	(Santos *et al*. 1998)
Crinum 'Ellen Bosanquet'	Micropropagation protocol from tri-scales.	The shoot formation happened in MS medium containing BA 22.2 μM. Explants were transferred and cultivated for 4 months in medium containing BA 35.5 μM for shoot stimulation and bulblet formation. They were then moved to free-GR medium, cultured for additional 3 months for finally be acclimatized and rooted *ex vitro*.	(Ulrich *et al*. 1999)
Zephyranthes L. sp. and	Micropropagation protocol for *in vitro* culture	*Zephyranthes* bulbs has challenges related to contamination of stage I cultures. Aseptically germinated seeds are a suitable source of clean bulb tissue. For *Hippeastrum* spp. bulbs resulted	(Smith *et al*. 1999)

Hippeastrum Herbert sp.	with bulb scales explants.	in contamination rates of 20-100%, and responded on a MS salt medium with NAA 2 mg L^{-1}.	
Narcissus pseudoNarcissus vars. Golden Harvest and St. Keverne	Development of a protocol for plant transformation using bulbs and shoot culture leaf explants.	Best medium to induce the somatic embryos (SEs) was MS + 2,4-D 5 µM and BA 0.5 or 5 µM. For the regeneration process, the SEs were cultivated in MS medium + IBA 4.95 µM at 4 °C.	(Sage *et al*. 2000)
Nerine sarniensis, Narcissus tazetta, Eucrosia radiate and *Haemanthus coccineus.*	Bud regeneration from inflorescence explants for micropropagation.	The medium used was MS mineral and organic constituents with specificities for each species: *Nerine sarniensis* (2.4-D 10 µM + BA 10 µM) and for *Narcissus tazetta, Eucrosia radiate* and *Haemanthus coccineu* (NAA 5 µM + BA 10 µM, with the addition of NaH_2PO_4 500 mg L^{-1}, adenine sulphate 100 mg L^{-1} and AC 2.5 g L^{-1}). Difco bacto agar (8 g L^{-1}) was used and the cultures were incubated at 25 ± 1°C, with 16/8h light/dark photoperiod, 50-60 µmol m^{-2} s^{-1}.	(Ziv and Lilien-Kipnis 2000)
Hyacinthus orientalis L. var. Carnegie	Regeneration protocol for *in vitro* culture with scale explants.	Treatment with IBA 1.5 and 3.0 mg L^{-1} showed higher regeneration and growth of the bulblets than with IAA at the same concentrations.	(Yi *et al*. 2002)
Cyrtanthus clavatus and Cyrtanthus spiralis	Development of micropropagation process and shoot growth techniques using twin-scales explants.	The culture medium employed was MS with charcoal 5 g L^{-1}, for the multiplication step, 2,4-D 0.1 mg L^{-1} and BA 1 mg L^{-1} produce the best number of shoots, and for the development of the bulbs the medium with NAA 1 mg L^{-1} and BA 2 mg L^{-1} was used. The best concentration of sucrose was 6% to induce roots and leaves.	(Morán *et al*. 2003)
Hippeastrum aulicum	Micropropagation protocol with floral and double-scale explants.	The bulb scale explants were more suitable than the floral explants, due to the elevated oxidation of the latter. The best culture media for shoot induction in bulb scale explants was MS with NAA 1,0 µM of and BA 4,0 µM and the treatments used for multiplication did not differ significantly.	(Flores 2003)
L. aestivum	Regeneration protocol using scales and leaves.	The regeneration occurred as a result of somatic embryogenesis in a media containing 2,4-D and picloram and by direct organogenesis on the medium rich in NAA.	(Ptak and Cierniak 2003)

Narcissus tazetta	Regeneration with twin-scales and inflorescence stems and culture conditions.	Twin-scale explants were established in solidified MS medium supplemented with sodium phosphate 2.5 mM and adenine sulfate 0.8 mM, NAA 5 µM, BA 10 µM and AC 5 g L^{-1}. For the inflorescence stem discs the AC was removed. The culture conditions were 25-22 ºC (day/night) and 16h day, wavelength at 70 µmol m^{-2} s^{-1}.	(Chen and Ziv 2005)
Narcissus tazetta L. *var. chinensis* Roem	Micropropagation of triploid species via callus and anthers.	The medium employed for the anthers was MS complemented with 2,4-D 0.5-1 mg L^{-1} and BA 0.5–2 mg L^{-1}, under dark conditions. The calli were initiated from anther connective tissue or anther wall tissue.	(Chen *et al*. 2005)
Galanthus nivalis, G. nivalis 'Flore Pleno' and *G. elwesii*	Method to stimulate growth of *in vitro*-produced bulblets from bulb scales explants, for acclimatization.	The explants were induced and maintained on MS medium supplemented with sucrose 30 g L^{-1}, BA 1 mg L^{-1} and NAA 0.1 mg L^{-1}. The treatments to induce the size of the bulblets were GR free and the combination of sucrose 30 or 60 g L^{-1} + AC 0, 1 or 5 g L^{-1}. Clump containing 2-4 bulblets were incubated for 16 weeks at 18°C with 16h photoperiod. Removing the GR didn't stop the bulb formation. AC promoted growth and induced root formation and elongation.	(Staikidou *et al*. 2006)
Hippeastrum x chmielii Chm.	Explore the effects of the growth retardant flurprimidol on the *in vitro* propagation of bulblets targeting the propagation rate, using twin-scales as explants. .	The bulblets used for the experiment were induced and cultivated in temporary immersion system (TIS) in MS medium + 6-benzylaminopurine (BA) 0.5 mg L^{-1} and NAA 0.1 mg L^{-1}, at pH 5.7. The TIS who presented the faster propagation was 8 immersions/ day for 15 minutes. The propagation rates increase significantly with flurprimidol 0.1 mg L^{-1}. The cultures were cultivated at 24 ±1 °C, 16 h of cool fluorescent light 5–10 µMol m^{-2} s^{-1} in shaken flasks (60 rpm).	(Ilczuk *et al*. 2005)
Hippeastrum hybridum	*Ex vitro* establishment of *in vitro* produced plantlets and bulblets.	To transfer the bulblets, an *in vitro* rooting step was not required. For the plantlets, this step was necessary. The medium used was MS supplemented by NAA 0.2 mg L^{-1}, with pH 5.8 and incubation at 24 ±1 °C, under 85% relative humidity, in a long day photoperiod cycle (16h).	(Siddique *et al*. 2007)

Narcissus L. var. Pink Charm	Protocol for *in vitro* culture from leaf explants.	The optimal medium was MS + BA 2 mg L^{-1} + NAA 1 mg L^{-1} for differentiation of rosette buds, MS + BA 1.5 mg L^{-1}+ NAA 1 mg L^{-1} for proliferation and ½ MS + BA 0.2 mg L^{-1} + NAA 0.5 mg L^{-1} for root induction.	(Zhu *et al*. 2007)
Rhodophiala montana	Bulb growth in different acclimatization conditions.	The plants were cultivated in MS medium, with BA 1 mg L^{-1} and NAA 0,1 mg L^{-1}, with 8 g $L^{-1}$of agar as gelling agent, in according to the protocols previously published (Schiappacasse *et al*. 2007).	(Jara *et al*. 2007)
Narcissus "fortissimo"	Rooting induction of bulblet in tissue culture seedling.	Strong roots grew in ½ MS + NAA 0.1 mg L^{-1} or IBA 0.1 mg L^{-1}. The bulblets grew faster on ½ MS + NAA 1.0 mg L^{-1} or IBA 0.5 mg L^{-1}. In ½ MS + NAA 0.1 mg L^{-1} or IBA 0.1 mg L^{-1} the bulbs had the significant promotion on the rooting induction.	(Cui 2008)
Rhodophiala montana (Phil.) Traub., Rhodophiala splendens (Rengifo) Traub., and Rhodophiala ananuca (Phil.) Traub.	Micropropagation process for preservation of this three Chilean native species.	The seeds germinated and formed the microbulbs. These were cultivated in MS liquid media with sucrose 30 g L^{-1} and kept at 22 ± 2 ºC under 50 µmol m^{-2} s^{-1} of light with a 16:8 h photoperiod. After acclimatization, they were transferred in a greenhouse at 21 ºC (min. 12 ºC; max. 29 ºC) with 15 h photoperiod and, placed into pots containing a soil/sand substrate (2:1) and cultivated for 7 or 8 week (fertilized every 15 d with KNO_3 1 g L^{-1} and NH_4PO_4 1 g L^{-1}). The microbulbs cultivated in smaller flasks develop 100% more fresh weight; with gelling agent, the hyperhydricity affected less plants and the survival rate was better during the acclimatization. The higher propagation rates in *R. ananuca* was reached with TIS.	(Muñoz *et al*. 2009)
Hippeastrum hybridum	Protocol for *in vitro* bulb production.	MS medium complemented with BA 6 mg L^{-1} and Chioro Choline Chloride (CCC) 500 mg L^{-1} fortified with sucrose 90 g L^{-1} resulted in the maximum bulb formation, with important weight, regeneration percentage and number of bulbs.	(Sultana *et al*. 2010)
Hymenocallis littoralis	Promote the effects of cytokinins for the shoot formation, using bulb scales as explants.	The culture medium was semi-solid MS with 2iP 13.5 µM for the shoot elongation, zeatin 2.25 µM for highest chlorophyll content or BA for shoot multiplication.	(Yew *et al*. 2010)

Narcissus var. *arkle.*	Micropropagation protocol for bulb production in a factory employing twin-scale explants.	The most appropriated medium was MS + BA 3.0 mg L^{-1} + NAA 0.5 mg L^{-1} + IBA 0.2 mg L^{-1}. For the multiplication step the GR changed (MS + BA 1.5 mg L^{-1} + NAA 0.3 mg L^{-1}) and for bulblet rooting the best cultivation medium was ½ MS + NAA 0.1 mg L^{-1} + AC 1.0 g L^{-1} reaching 80% of rooting.	(Sun *et al*. 2010)
Narcissus L. var. Delibes	Shoot induction, bulbil formation and root differentiation.	The two optimum media for bulbil formation were: MS supplemented with BA 4 mg·L^{-1}+ NAA 0.2 mg·L^{-1}, activated charcoal (AC) 2 mg·L^{-1} and sucrose 60 g·L^{-1} or MS with BA 2 mg·L^{-1}, 2,4-D 1 mg·L^{-1}, AC 2 mg·L^{-1} and sucrose 90 g·L^{-1}. Roots could be induced in MS with BA 1 mg·L^{-1}, 2,4-D 0.5 mg·L^{-1}, NAA 0.5 mg·L^{-1}, AC 2 mg·L^{-1} and sucrose 30 g·L^{-1}.	(Lv *et al*. 2010)
Narcissusin suzhou	Protocol for *in vitro* culture with twin-scales.	MS medium plus BA 3.2 mg L^{-1} + NAA 0.02 mg L^{-1} was more efficient to induce the small bulbs in about 4-5 weeks. The growth rate was up to 322.22%.	(Jiang *et al*. 2010)
Pancratium. maritimum	Protocol for induction of friable callus and shoot cultures using young fruits as explants.	For callus induction, MS was supplied with BA 2.0 mg L^{-1} and 2,4-D 1.0 or 4.0 mg L^{-1}. For shoot regeneration was used MS with sucrose 30 g L^{-1}, agar 5.5 g L^{-1}, NAA 1.15 mg L^{-1} and BA 2.0 mg L^{-1}. Growth conditions were 16/8h photoperiod, with light intensity of 110 µmol m^{-2} s^{-1}, at 26°C.	(Georgiev *et al*. 2010)
Brunsvigia undulata F.M. Leight	Micropropagation protocol for *in vitro* bulblet production from twin-scale explants.	The inoculation occurs on MS medium with the addition of *myo*-inositol 0.1 g L^{-1}, agar 8 g L^{-1}, sucrose 30 g L-1. Cultures were kept at 25 ±1 °C, 16 h photoperiod, intensity of 74.4 µmol m^{-2} s^{-1}. Bulblets were formed using almost all the treatments, including the GR-free medium (100 % of the explants). Twin-scales were excised from the middle of the parent bulb, placed adaxial side down on GR-free medium and kept in a 16 h photoperiod.	(Rice *et al*. 2011)
Cyrtanthus 'Orange Gem' × *C. eucallus* hybrid	*In vitro* propagation with different explants and effect of GR on	Single-scale explants with the basal plate tissue attached were cultured on MS medium supplemented with BA 2.5 mg L^{-1} + NAA 0.5 mg L^{-1}, sucrose 90 g L^{-1}, agar 5 g L^{-1}, pH 5.8. Growth chamber temperature 22 ºC (first 3 days) and 27 ºC, under 24 h light at 19 µE s^{-1} m^{-1}.	(Hong and Lee 2012)

	bulblet multiplication.		
Galanthus nivalis 'Flore Pleno'	*In vitro* bulblet multiplication and growth.	The process was evaluated through three 16-week sub-culture passages on G media (Staikidou *et al*. 2006) supplemented with sucrose 60 g L^{-1} and AC 5 g L^{-1}, to reduce the bulblet multiplication and growth (FW).	(Staikidou and Selby 2012)
Hymenocallis littoralis (Melong kecil)	Callus induction employing bulbs and root explants.	The best callus formation was obtained using root explants on MS basal media, with 3% (w/v) sucrose and 0.25% (w/v) gelrite plus NAA 2 mg L^{-1}. For bulb explants the best GR was 2,4-D 2.0 mg L^{-1}. The pH of the medium was adjusted to 5.7 to 5.8, and the cultures were incubated at 25 ± 2°C in the dark for 4 weeks to initiate callogenesis.	(Noormi *et al*. 2012)
Narcissus tazetta var.chinensis	Calli induction and organogenesis.	The hormone 2,4-D used in higher concentration (3.0 to 4.0 mg L^{-1}) + BA induce colorless embryogenic calli. Using moderate concentration of 2,4- D (0.5-1 mg L^{-1}) the production of shoot buds was stimulated. The calli induction and organogenesis are auxin-type dependent and related with their concentrations in the medium.	(Fang *et al*. 2013)
L. aestivum	*In vitro* bulbs induction and acclimatization	MS medium supplemented with zeatin 5 μM and BA 0.5 μM (Ptak *et al*. 2013a; Ptak *et al*. 2013b) was used. With sucrose 90 g L^{-1} or paclobutrazol the highest fresh weight and best survival rate (100%) were obtained. When ancymidol was added the bulbs presented the highest number of leaves and roots. With the system RITA®, the plants had a successful *ex vitro* adaptation.	(Ptak 2014)
Galanthus nivalis L.	Shoot and plant regeneration using bulb scale-.	MS basal medium with Gamborg vitamins (Gamborg *et al*. 1968), sucrose 20 g L^{-1}, agar 8 g L^{-1} and GR. Cultures were maintained at 14/10 (light/dark) photoperiod (white fluorescent light, 10 μmol m^{-2} s^{-1}) and 22/18 ± 2 °C. The GR used were NAA 2 mg L^{-1} + IAA 2 mg L^{-1} or NAA 2–10 mg L^{-1} with BA 1 mg L^{-1}. A cytokinin on full-strength media was required for regeneration, and the induction and maintenance with NAA 2 mg L^{-1} and BA 1 mg L^{-1}, produced mature/imature bulblets with shoots.	(Resetár *et al*. 2014)
Traubia modesta	Establish a protocol for *in vitro* culture using twin scales from	The twin scales were inoculated in MS medium with NAA and BA with no significant differences between the treatments. A multiplication rate of 1.3 – 2.2 for bulbils was achieved, with an average of 28 bulbils per mother bulb, compared to 1 to 4 bulbils per mother bulb/ year in wild plants.	(Paredes *et al*. 2014)

	bulbils as explants.		
Cyrtanthus contractus, Cyrtanthus guthrieae, and *Cyrtanthus obliquus*	Development of *in vitro* regeneration systems, using twin-scale explants.	The twin scales were cultured on solid MS under 16/8-h light/dark conditions at 25±2°C. The best shoot induction responses were obtained using BA 4.4 µM + NAA 1.1 µM for *C. contractus* and *C. guthrieae* and BA 6.7 µM + NAA 2.7 µM for *C. obliquus*.	(Ncube *et al*. 2015)
Hippeastrum hybridum	Micropropagation protocol using single scale explants.	NL medium with 2,4-D 5 mg L^{-1} produce the major quantity of bulblets.	(Amani *et al*. 2015)
Cyrtanthus mackenii Hook	*in vitro* production of bulbs from tri-scales.	The explants cultured on MS medium supplemented with TDZ 1 mg L^{-1} and NAA 1 mg L^{-1} produced a maximum of bulblets. The maximum number and length of roots was obtained using TDZ 1 mg L^{-1} + NAA 1 mg L^{-1}. Sucrose at 60 g L^{-1} increased the size of bulblets after three months of culture. Bulblets were then transferred to pots with vermiculite: soil (3:1) with 98% survival.	(Joseph *et al*. 2016)

Abbreviations: 2-isopentenyladenine (2iP); 1-naphthaleneacetic acid (NAA); 6-benzylaminopurine (BA); 2,4-dichlorophenoxyacetic acid (2,4-D); growth regulators (GR); indole-3-butyric acid (IBA); Electron microscopy (EM); Activated Charcoal (AC); Fresh Weight (FW); Indole-3-acetic acid (IAA); paclobutrazol (PAC); Chioro Choline Chloride (CCC); Somatic embryos (SEs); Temporary Immersion System (TIS).

Table 3. Amaryllidaceae alkaloids produced by *in vitro* techniques (plant cell, tissue and organs cultures).

Species	Alkaloid produced	Culture type	Culture conditions	Reference
Narcissus confusus	Galanthamine (GAL)	*In vitro* shoot-clumps culture in liquid medium.	The shoot-clumps were transferred to MS (Murashige and Skoog 1962) liquid medium (LM) without growth regulators (GR) and sucrose 60 g L^{-1} at 120 rpm. After almost 2 months, the roots were induced and the shoots transferred to soil. The analysis of alkaloid content revealed that the calli do not present these secondary metabolites, but released them in the LM, which is ideal for bioreactor production. The culture with the *trans*-cinnamic acid 1 g L^{-1} precursor induce the release of GAL in the medium.	(Fuster 1994)
N. confusus	GAL and *N*-formyl-norgalanthamine.	Shoot-clumps culture.	Using twin-scales cuts as explants, the medium for bud proliferation used was MS + 2,4-dichlorophenoxyacetic acid (2,4-D) 1 mg L^{-1} and 6-benzylaminopurine (BA) 5 mg L^{-1} in liquid shaked medium.	(Bergonon *et al*. 1996)
N. confusus	GAL.	Shoot-clumps cultures.	Shoot-clumps were induced from buds obtained from twin-scales and from organogenic calli on a MS medium supplemented with 2,4-D 1 mg L^{-1} and BA 5 mg L^{-1}. To develop the shoot-clumps they were kept partially submerged in a LM (110 rpm) and then, they were transferred to a medium with sucrose 90 g L^{-1}. The photoperiod was 16 h light/8 h dark. The sucrose can affect the alkaloid profile and the alkaloids excreted in the medium.	(Sellés *et al*. 1997)

N. confusus	GAL.	Callus induction, somatic embryogenesis and organogenesis.	Callus were induced from seeds in MS medium + sucrose 30 g L^{-}1, difco-agar 8 g L^{-1} and pH 5.7, maintained at 25 ±1 ºC under a long-day photoperiod, for 3 months. The embryos were excised from seeds and placed in callus induction medium (MS + 2.4-D or 4-amino-3,5,6-trichloropicolinic acid (Picloram) 10 mg L^{-1}), with the same light regime. Germinated somatic embryos were shifted to MS + BA or Kinetin (Kin) 0.5 or 1 mg L^{-1} during 3 months. When the clumps grew, they were transferred to MS + 2,4-D 1 mg L^{-1} and BA 5 mg L^{-1} in solid medium. The undifferentiated calli produced small amounts of GAL, which increased with the degree of tissue differentiation.	(Sellés *et al*. 1999)
Crinum moorei	Cherylline, crinamidine, crinine, epibuphanisine, lycorine (LYC), powelline, undulatine, 1-epideacetyl-bowdensine and 3-O-acetylhamayne.	*In vitro* propagated bulbs.	The bulbs were induced from twin-scales in MS medium with 16 h photoperiod, 70 µmol m^{-2} s^{-1} light intensity and 25 ºC temperature. BA and Activated Charcoal (AC) positively influence the levels of specific alkaloids in both the bulblets and media culture.	(Fennell *et al*. 2003)
N. confusus	GAL.	Shoot-clumps	The shoot clumps were cultivated in MS liquid medium + BA 3 mg L^{-1} and Plant Preservative Mixture (PPM^{TM}) 0.5 ml L^{-1} and Methyl jasmonate (MJ) 25 µM in rotatory shaker at 110 rpm and 25 ºC, with 16h light photoperiod, and light intensity of 100 µmol m^{-2} s^{-1}.	(Colque *et al*. 2004)

Leucojum aestivum L.	GAL, LYC and crinane types (11 in wild plants and eight in the *in vitro* cultures).	Wild plants and *in vitro* cultures	The organs used as explants were bulbs, leaves and ovaries. The bulblets were cultivated in BA 2 mg L^{-1} and 1-naphthaleneacetic acid (NAA) 0.15 mg L^{-1} and for the shoot clumps, the GR were changed for Kin 2 mg L^{-1} and 2,4-D 0.15 mg L^{-1}, on a rotary shaker at 110 rpm; both at 25 ± 2°C and 13/11 h photoperiod.	(Berkov *et al*. 2005)
L. aestivum	GAL.	*In vitro* cultures in different stages of morphogenesis	The best results were found with bulblets grown in MS medium with NAA 10 µM and BA 0.5 µM. Bulblets were regenerated by indirect organogenesis, from the leaves.	(Diop *et al*. 2006)
L. aestivum	GAL.	Hairy root and bulblets.	MS medium + NAA 10 µM and BA 0.5 µM. Regeneration by direct organogenesis.	(Diop *et al*. 2007)
L. aestivum	GAL, LYC, haemantham ine and homolycorin e type alkaloids.	Shoot-clumps.	The *in vitro* cultures were initiated from bulbs in solid B5 medium (Gamborg *et al*. 1968) with sucrose 30 g L^{-1}, agar 6 g L^{-1}, and kept in darkness. Casein hydrolysate 0.5 mg L^{-1}, 2,4-D, 1 mg L^{-1}, adenine 2 mg L^{-1}, and glutathione 10 mg L^{-1} were added to the starting culture medium. The shoots were transferred to MS medium supplemented with $Ca(NO_3)_2$ 1 g L^{-1}, BA 0.5 mg L^{-1} indole-3-butyric acid (IBA) 0.01 mg L^{-1} and paclobutrazol 2.93 mg L^{-1}; at 24 ± ºC with 16h light.	(Georgieva *et al*. 2007)
L. aestivum	GAL.	Shoot cultures.	The callus induction from young fruits happened in MS medium plus 2.4-D 4 mg L^{-1} and BA 2 mg L^{-1}. Calli were transferred to the same nutrient medium with NAA 1.15 mg L^{-1} and BA 2.0 mg L^{-1} to stimulate the differentiation in shoots. The maximum yield of biomass and amount of GAL were achieved in the shoot cultures after 35 days of submerged cultivation at 110 rpm, under light. Accumulation of GAL was strongly dependent on the level of differentiation.	(Pavlov *et al*. 2007)

L. aestivum	GAL, LYC and other 24 alkaloids.	Intact plants, calli and shoot-clump cultures.	For callus cultures initiated from young fruits, the medium used was MS + 2,4-D 4 mg L^{-1} and BA 2 mg L^{-1}. The shoot-clumps grew from callus in MS + NAA 1.15 mg L^{-1} and BA 2 mg L^{-1} under light.	(Berkov *et al*. 2009)
L. aestivum	GAL and LYC.	*In vitro* clonal propagation.	MS medium + 10x thiamine-HCl + sucrose 30 g L^{-1}, BA 2 mg L^{-1} and NAA 0.15 mg L^{-1}, at 23 ºC and 16/8h photoperiod.	(Bogdanova *et al*. 2009)
L. aestivum	GAL.	*In vitro* shoot system.	Modified MS nutrient medium, with KNO_3 4.50 g L^{-1}, NH_4NO_3 0.89 g L^{-1}, $(NH_4)_2SO_4$ 1.25 g L^{-1}, KH_2PO_4 0.10 g L^{-1} and sucrose 60 g L^{-1}, to promote increased production of GAL in shoot cultures from calli. The illumination used was 16h light/8h darkness per day, at 26 ºC.	(Georgiev *et al*. 2009)
L. aestivum	GAL and LYC.	Callus and *in vitro* cultures.	Leaves explants were cultivated in MS medium with picloram 25 µM and BA 0.5 µM, $KMnO_4$ 4.5 g L^{-1}, sucrose 30 g L^{-1}, pH 5.5 and agar 8 g L^{-1}, at 25 ± 2 ºC, in the dark. The maximum GAL and LYC concentrations and highest diversity of alkaloids were obtained in the presence of $KMnO_4$.	(Ptak *et al*. 2009)
L. aestivum	GAL and LYC.	Shoot-clumps generated by direct organogenesis.	Crops grown in MS based medium with a 10-fold increased thiamine-HCl, pH 5.7, agar 6 g L^{-1} supplemented with BA 2 mg L^{-1}, NAA 0.15 mg L^{-1} and sucrose 30 g L^{-1}, cultivated at 23 ±2 °C, 16/8 h light/dark period and illumination of 20.25 µmol m^{-2} s^{-1}. This shoot-clump cultures demonstrated high biosynthetic capacity, GAL and LYC ratios and content were genotype and age-dependent. Addition of the precursors tyrosine and phenylalanine in the medium enhanced GAL and LYC production.	(Stanilova *et al*. 2009)

L. aestivum	D_3-galanthamine, crinine and demethylmarithidine.	Shoot cultures deuterium-labeled.	The initial explants were cultivated on MS medium + NAA 10 µM and BA 0.5 µM, sucrose 3 %, pH 5.5 and agar 0.8 %. The culture was done at 25 ± 2 ºC in the dark. The total of six labeled alkaloids were identified after the addition of the precursor, 4'-*O*-methyl-d_3-norbelladine, to the medium.	(El Tahchy *et al*. 2010)
L. aestivum	GAL.	Embryo cultures.	MS medium + 25 µM Picloram and 0.5 µM BA for callus induction. When $AgNO_3$ (inhibitor of ethylene) was added to the medium, callus growth increased and after the addition of $KMnO_4$ (an absorbent) the plants developed better. For the GAL production in callus, the best inductor was Silver thiosulphate (STS), and for somatic embryo cultures, the 1-aminocyclopropane-1-carboxylic acid (ACC).	(Ptak *et al*. 2010)
L. aestivum	GAL and LYC.	Shoot-clumps by direct organogenesis.	Bulbs and shoots cultivated in MS medium + BA and NAA, under illumination provided by fluorescent tubes or in the dark at 25 ºC.	(Stanilova *et al*. 2010)
Pancratium maritimum	Homolycorine and tazettine types, GAL.	Calli, shoot-clumps and regenerated plants.	Young fruits were used for the induction of the cultures. The medium for callus formation was MS supplemented with 2,4-D 1 or 4 mg L^{-1} and BA 2 mg L^{-1}. For shoot initiation and organogenesis, the calli and the slices of fruit were cultivated in MS medium + NAA 1.15 mg L^{-1} and BA 2 mg L^{-1}.	(Berkov *et al*. 2010)

Hippeastrum vittatum	Ismine, trisphaeridine, GAL , sanguinine, vitattine, 8-*O*-demethylmaritidine, anhydrolycorine, 11,12-dehydroanhydro-lycorine, montanine, pancracine, 11-hydroxyvittatine, LYC, pseudolycorine.	*In vitro* bulb culture.	To induce multiplication, the medium was supplemented with sucrose 30 g L^{-1}, 2-isopentenyladenine (2iP) 16 mg L^{-1} + NAA 4 mg L^{-1}, spermine 80 mg L^{-1} (best multiplication rate) and methyl jasmonate 4 mg L^{-1} (highest bulb fresh weight). The cultures were incubated at 25 ±2 ℃ under dark. The bulbs were successfully rooted on MS with NAA 4 mg L^{-1} and presented 10% of survival when acclimatized. Chemical analysis showed changes in the alkaloid type ratio and number of compounds when the bulbs were treated with methyl jasmonate 4 mg L^{-1}.	(Zayed *et al*. 2011)
L. aestivum	GAL and LYC.	Shoot cultures	MS liquid medium + NAA 10 µM and BA 0.5 µM. The better production of GAL was found when the precursor 4'-*O*-methylnorbelladine (MN) was added at 0.10 g L^{-1} to the medium. For LYC, the best concentration of MN was 0.20 g L^{-1}.	(El Tahchy *et al*. 2011b)
L. aestivum	GAL, LYC and norgalanthamine.	Shoot culture in temporary immersion	The shoot cultures were established by calli cultures in MS nutrient medium, supplemented with sucrose 30 g L^{-1}, NAA 1.15 mg L^{-1}, BA 2.0 mg L^{-1} and "Plant agar" 5.5 g L^{-1} (Georgiev *et al*. 2009). The maximal yield of GAL was achieved in temporary immersion RITA® system with the following immersion frequency: 15 min flooding and 8 h stand-by periods, at 26 ℃, 16 h light/8 h dark per day. The flow rate of the inlet air was 60 l/h for each RITA® apparatus.	(Ivanov *et al*. 2011)

Narcissus pseudoNarcissus, *Galanthus elwesii* and *L. aestivum*	GAL, trisphaeridine, anhydrolycorine, crinine, demethylmaritidine and narwedine.	Shoot cultures.	The first medium was MS nutrient medium, pH 5,5 before autoclaving (120 ºC), with sucrose 60 g L^{-1} and BA 4 μM with 2,4-D 10 μM were optimal for callus formation; and BA 5 μM with 2,4-D 12 μM for bulb formation. All cultures were maintained at 25 ± 2°C, under a 16/8-h (light/dark) photoperiod. The highest diversity was reached with *G. elwesii* shoots cultured on MS + NAA or picloram 10 μM. The best alkaloid variability was obtained from *N. pseudoNarcissus* shoots cultured on MS + NAA 10 μM. *L. aestivum* had the lowest diversity.	(El Tahchy *et al*. 2011a)
P. maritimum	Tyramine, narciclasine, GAL, haemanthamine, LYC, pancracine, tazettine and homolycorine types (22 compounds)	Shoots in liquid culture.	Young fruits were used as explants following the published protocol (Georgiev *et al*. 2010). For the liquid medium, the MS medium was used in the same concentration of sucrose, NAA and BA, in shaker (100 rpm) and in the same light conditions.	(Georgiev *et al*. 2011)
L. aestivum	GAL.	Shoot culture in a modified bubble column bioreactor.	The initial shoot cultures were grown for 28 days in MS solid medium with sucrose 30 g L^{-1}, agar 5.5 g L^{-1} and NAA 1.15 mg L^{-1} (Georgiev *et al*. 2010). The medium used for bioreactor cultivation was MS, 16/8h photoperiod, with light intensity of 110 μmol m^{-2} s^{-1} cultured at 22°C and 18 L/(L·h) flow rate of inlet air for the highest amounts of dry mass and GAL.	(Georgiev *et al*. 2012)
L. aestivum	GAL, hamayne, LYC and other 15 alkaloids.	Shoot culture in temporary immersion.	The shoot culture was obtained by indirect organogenesis (Georgiev *et al*. 2009; Ivanov *et al*. 2011) and cultivated for 35 days in RITA® with 200 mL optimized medium for each 12g fresh shoots, same illumination conditions and	(Ivanov *et al*. 2012)

			flow rate of the inlet air of 60 Lh^{-1} at 26 ºC.	
L. aestivum	GAL.	Shoot cultures.	The medium used was MS with some modifications in the vitamins (glycine 40 mg L^{-1}, nicotinic acid 10 mg L^{-1}, pyridoxine HCl 1 mg L^{-1}, thiamine HCl 30 mg L^{-1}) and BA 2 mg L^{-1} + NAA 0.15 mg L^{-1}. The maximum yield of GAL was found when the shoots were cultured in 1-liter bioreactor vessel, with 12 times per day a temporary immersion system of 5 minutes.	(Schumann *et al*. 2012)
L. aestivum	GAL and LYC.	Liquid shoot cultures	The shoot cultures were obtained using the protocol published by Pavlov *et al*. (2007). The medium used for elicitation was MS, with the culture at 26 ºC on a shaker (110 rpm) under illumination with a photoperiod of 16 h light/8 h dark. The addition of elicitors was during the exponential phase of the culture growth and the best alkaloid content was achieved using jasmonic acid for the induction of tyrosine decarboxylase.	(Ivanov *et al*. 2013)
L. aestivum	GAL and LYC.	Plants regenerated from somatic embryos.	MS medium + meta-topolin and BA regenerate the largest number of plants. The highest biomass and content of GAL was obtained in RITA ® with plants in presence of Thidiazuron (TDZ). For LYC, the plants in solid media with the addition of TDZ was better.	(Ptak *et al*. 2013a)
L. aestivum	GAL, trisphaeridine, tazettine, and 11-hydroxyvittatine.	Somatic embryos.	Initial explants were cultivated in MS medium with BA 0.5 µM, sucrose 30 g L^{-1}, pH 5.5 and agar 8 g L^{-1}. The induction of embryos in the explants was for dicamba 25 µM (30% of response); picloram 25 and 50 µM (100%). 2,4-D 50 µM stimulated greater than the other auxins callus proliferation and somatic embryo formation. GAL was detected in callus cultivated with 2,4-D 25 and 50 µM, picloram 25 µM and dicamba 50	(Ptak *et al*. 2013b)

			µM. Other alkaloids, trisphaeridine, tazettine, and 11-hydroxyvittatine were accumulated only in callus growing on medium with 50 µM picloram.	
L. aestivum	GAL.	Shoot cultures in different bioreactors systems.	MS basal medium supplemented with *myo*-inositol 100 mg L^{-1}, nicotinic acid 10 mg L^{-1}, pyridoxine hydrochloride 1 mg L^{-1}, thiamine hydrochloride 30 mg L^{-1}, glycine 40 mg L^{-1}, sucrose 30 g L^{-1}, BA 2 mg L^{-1}, NAA 0.15 mg L^{-1}, gelrite 3 g L^{-1}, pH 5.8 before autoclaving at 121 °C for 20 min. Cultures maintained at 24 ±2 °C, 16/8 h (light/dark) photoperiod, photosynthetic photon flux of 30µM m^2 s^{-1}. The shoots were used as explants and the maximal yield of GAL was achieved by cultivating the shoots in a temporary immersion system in a 1-L bioreactor vessel which was used as an airlift culture vessel, gassing 12 times per day (5 min).	(Schumann *et al*. 2013)
Crinum americanum	Alkaloids.	Cell cultures.	The callus induction was realized on MS medium supplemented with 3% sucrose, 0.8% agar, pH adjusted to 5.8 and 2,4-D 2.26 µM. The flasks were kept in a chamber (BOD Eletrolab) for 30 days in the dark at 24°C. After the initial analysis, the results suggested the presence of alkaloids.	(Araújo and Santos 2014)
Cyrtanthus guthrieae L.	Total alkaloids.	Callus-derived plants.	MS solid medium plus different concentrations of carbon and nitrogen. The alkaloid accumulation became ideal with sucrose 15 mM, NH_4NO_3 41.3 mM and KNO_3 37.6 mM.	(Ncube *et al*. 2014)
Hymenocallis littoralis	LYC.	Callus cultures.	To initiate callus cultures, the meristematic tissues of the bulb were inoculated in MS media, under dark condition at 25 ± 2 °C. The highest LYC content was found in the bulb extract and the least in the root extract of the wild plants. Few callus culture samples had high content of LYC, comparable to that	(Subramaniam *et al*. 2014)

			of wild plants. The results showed that the best GR in the medium to increase the LYC content was 2,4-D 4.5 μM and the combination of 2,4-D 9.00 μM + with BA 4.5 μM in callus culture.	
Narcissus tazetta var. italicus	Total alkaloids.	Tissue cultures.	Bulb and leaves explants were cultivated in MS supplemented with BA 1.5 mg L^{-1} and NAA 3 mg L^{-1}; BA 2 mg L^{-1} and Indole-3-acetic acid (IAA) 0.5 mg L^{-1}. BA 2 mg L^{-1} and IBA 1mg L^{-1} generated bulblets derived from bulb explants. The fungi, *Fusarium sporotrichioides*, was the most effective elicitor in enhancing growth and total alkaloids when incubated on MS medium + BA 1.5 mg L^{-1} and NAA 3 mg L^{-1} for 10 days with 4 weeks old callus cultures.	(Taleb *et al*. 2014)
Pancratium maritimum.	LYC.	Bulblets.	MS based medium with BA 3 mg L^{-1} and NAA 0.1 mg L^{-1}; room culture temperature at 23 ±2 ℃, photoperiod 16/8 light/dark and agar 6 g L^{-1}, for the cultivation of bulblets regenerated by direct organogenesis from mature seeds.	(Bogdanova *et al*. 2014)
P. maritimum	Narciclasine, GAL, haemanthamine, LYC, montanine, tazettine, homolycorine and tyramine types (22 compounds)	Temporary immersion for shoot culture	The MS medium and light conditions were those published in (Georgiev *et al*. 2010). The maximum amount of biomass and alkaloids production were found with immersion frequency of 15 min flooding and 12 h stand.	(Georgiev *et al*. 2014)
L. aestivum	GAL and LYC.	Shoot cultures.	The precursor MN was incorporated into the LM at the concentration of 0.1 g L^{-1} to elicit the shoot cultures, during 15 days of culture. After the alkaloid extraction the results showed that the precursor highly stimulated the biosynthesis of both	(Saliba *et al*. 2015)

			GAL and LYC after its biotransformation by the shoot cultures.	
L. aestivum and *L. aestivum* 'Gravety Giant'	GAL and LYC.	Bulblets cultivated in temporary immersion conditions.	The bulb cultures were subcultured in medium containing the precursor MN and cultivated in bioreactor RITA®. The precursor feeding along with TI conditions improved the alkaloids accumulation and the maximal concentrations of GAL was found after 40 days of culture and for LYC after 30 days of culture with MN 0.3 g L^{-1} in *L. aestivum* bulblets. In *L. aestivum* 'Gravety Giant' bulb cultures, MN 0.3 g L^{-1} was the best condition for GAL, after 50 days and for LYC after 30 days of culture.	(Saliba *et al*. 2016)

Abbreviations: 1-aminocyclopropane-1-carboxylic acid (ACC); 1-naphthaleneacetic acid (NAA); 2-isopentenyladenine (2iP); 2,4-dichlorophenoxyacetic acid (2,4-D); 4-amino-3,5,6-trichloropicolinic acid (Picloram); 4'-*O*-methylnorbelladine (MN); 6-benzylaminopurine (BA) Activated Charcoal (AC); Galanthamine (GAL); Growth Regulators (GR); Indole-3-acetic acid (IAA); indole-3-butyric acid (IBA); Kinetin (Kin); Liquid Medium (LM); Lycorine (LYC); Methyl Jasmonato (MJ); Plant Preservative Mixture (PPM^{TM}); Silver thiosulphate (STS); Thidiazuron (TDZ).

Table 4. Amaryllidaceae plant protocols using genetic transformation.

Species	Main of the study	Strain	Culture conditions	Reference
Narcissus cv.Paperwhite	It is possible that the Ti plasmid can be useful as vector for transforming moncotyledoneous plant species.	*Agrobacterium tumefaciens* strain LBA2347	Not shown, but the results prove that after 21 days the infected plants develop small swellings at the infection site and are positive for the presence of nopaline, a positive control for the infected cells.	(Hooykaas-Van Slogteren *et al*. 1984)
Narcissus pseudoNarcissus var. Golden Harvest and St. Keverne	Somatic embryogenesis and transformation protocol employing scale explants.	*A. tumefaciens* wild and engineered strain (not showed)	The highest yields of direct somatic embryos (SEs) were obtained in MS-based medium (Murashige and Skoog 1962). SEs were converted to plantlets on a medium with indole-3-butyric acid (IBA) in a temperature dependent manner. Plantlets could be transferred to *ex vitro* conditions. Nodular calli were produced from Golden Harvest and St. Keverne, on a medium with thidiazuron (TDZ) + 1-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D). The callus can grow in a liquid medium as well as transformed material via *A. tumefaciens*, wild and engineered strain.	(Sage and Hammatt 2002)
Hippeastrum x *chmelii* Chm..	Stablish protocol for selecting transgenic cells/ regenerated with young flower stems.	*Agrobacterium* -mediated transformation system (strain not shown).	The medium for bulb regeneration was MS salts, MS vitamins lacking peptone, 2-isopentenyladenine (2iP) 2.0 mg L^{-1}, NAA 0.2 mg L^{-1}. For the second propagation system the explants were cultured on MS medium + BA 0.5 mg L^{-1} and NAA 0.1 mg L^{-1} at 25 °C, 30 µMol m^{-2} s^{-1} for 16 hours per day. The higher rates for later selection of the cells expressing *PAT* gene were found in MS+ glufosinate 2.0 mg L^{-1}.	(Ilczuk *et al*. 2006)
L. aestivum L.	Hairy roots system culture.	*Agrobacterium rhizogenes*	MS medium + NAA 10 µM and BA 0.5 µM to induce the cultures with	(Diop *et al*. 2007)

		strain LBA 9402.	thin slices of leaves and to maintain the *in vitro* cultures.	
Narcissus tazzeta var. chinensis	Phytoene synthase (*PSY*) gene, enzyme for carotene biosynthesis.	*Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector pCAMBIA1301.	Bulbs scales explants were transformed in selected medium MS + BA 5 mg L^{-1} + NAA 0.1 mg L^{-1} and hygromycin 40 mg L^{-1} for regenerated plants.	(Lu *et al*. 2007)

Abbreviations: 1-naphthaleneacetic acid (NAA); 2-isopentenyladenine (2iP); 4-dichlorophenoxyacetic acid (2,4-D); 6-benzylaminopurine (BA); indole-3-butyric acid (IBA); Somatic embryos (SEs); temporary immersion system (TIS); thidiazuron (TDZ).

Supplementary material.

Table SM1. Salt based medium employed in the studies for micropropagation of Amaryllidaceae plants.

Medium	Percent
1/2 MS	12,28
G media	1,75
Gamborg vitamins	1,75
MMS	1,75
MS	78,95
N6	1,75
NL	1,75

Table SM2. Quantity of sucrose added to the medium culture for micropropagation of Amaryllidaceae plants.

Sucrose	Percent
30	40,91
60	31,82
90	18,18
20	9,09

Table SM3. Plant growth regulators used in the induction of diverse *in vitro* explants related with the quantity of citations in the literature.

Chemical compound	Use	Concentration mg L^{-1}	References cited	% total
BA	bulblets/calli/shoots/rooting	1,0	15	8,5
BA	bulblets/calli/shoots	2,0	14	8,0
NAA	bulblets/mult/rooting/shoots	0,1	13	7,4
NAA	bulblets/shoots	1,0	11	6,3
NAA	calli/bulblets/ multiplication and induction/ rooting/shoots	0,5	10	5,7
2,4-D	bulblets/calli/shoots/embryos	1,0	6	3,4
BA	bulblets/shoots	4,0	6	3,4
Charcoal	shoot	5,0	6	3,4
NAA	calli/shoots induction and maintenance	2,0	6	3,4
BA	calli	2,3	5	2,8
NAA	calli/bulblets/shoots	0,2	5	2,8
2,4-D	shoots/bulblets/calli	0,1	4	2,3
2,4-D	calli	2,0	4	2,3
2,4-D	calli/rooting/shoots	0,5	3	1,7
BA	bulblets/calli	0,5	3	1,7
BA	bulblets multipl/shoots	1,5	3	1,7
BA	bulblets	3,0	3	1,7
Zeatin	bulblet/protocorm/shoot	1,0	3	1,7
IAA	bulblet/shoot	1,0	3	1,7
2,4-D	calli	4,0	2	1,1
BA	bulblets	3,2	2	1,1
BA	shoots ind and maintenance/multiplication	5,0	2	1,1
BA	bulblets	6,0	2	1,1
BA	bulblets	8,0	2	1,1
Charcoal	bulblet	2,0	2	1,1
Charcoal	rooting/shoot	2,5	2	1,1
IBA	rooting	0,1	2	1,1
NAA	rooting/shoots	1,2	2	1,1
TDZ	bulblets	1,0	1	0,6
TDZ	rooting	1,0	1	0,6
CCC	bulblets	500,0	1	0,6
Kinetin	shoots	0,1	1	0,6
Kinetin	shoots	1,0	1	0,6
2,4-D	calli	3,0	1	0,6

2,4-D	bulblets	5,0	1	0,6
BA	embryo	0,1	1	0,6
BA	embryos	0,1	1	0,6
BA	rooting	0,2	1	0,6
BA	embryos	1,1	1	0,6
BA	bulblets multipl	2,5	1	0,6
Paclobutrazol	calli	-	1	0,6
Charcoal	bulblet	1,0	1	0,6
Charcoal	rooting	0,1	1	0,6
IBA	bulblets	3,0	1	0,6
IBA	bulblets	1,5	1	0,6
IBA	bulblets	1,1	1	0,6
IBA	bulblets	0,5	1	0,6
IBA	bulblets	0,4	1	0,6
IBA	shoots	1,0	1	0,6
IBA	shoots	1,0	1	0,6
Ancymidol -A-Rest ™	shoots	1.25	1	0,6
2iP	bulblets	2,0	1	0,6
2iP	shoot elongation	2,7	1	0,6
NAA	bulblets	0,0	1	0,6
NAA	bulblets	0,0	1	0,6
NAA	bulblets multiplication	0,3	1	0,6
NAA	calli	0,0	1	0,6
Zeatin	embryo	0,1	1	0,6
IAA	bulblet	4,7	1	0,6
IAA	bulblet	3,0	1	0,6
IAA	bulblet	1,5	1	0,6
IAA	shoot	2,0	1	0,6
IAA	shoot	0,1	1	0,6

Table SM4. Medium culture used in the induction of Amaryllidaceae alkaloids in *in vitro* cultures according with the literature analysis.

Medium	Citations	Percent
B5	1	2,9
MS	34	97,1

Table SM5. Physical state of the culture medium used in the induction of Amaryllidaceae alkaloids in *in vitro* cultures according with the literature analysis

Physical condition	Citations	Percent
Solid	18	50
Liquid	18	50

Table SM6. Sucrose concentration in the culture medium used for Amaryllidaceae alkaloids induction in *in vitro* cultures according with the literature analysis.

Sucrose	Citations	Percent
30	13	72,2
60	3	16,7
90	1	5,6
5	1	5,6

Table SM7. Types of organs used in the Amaryllidaceae alkaloids production in *in vitro* cultures according with the literature analysis.

Organ/tissue	Citations	Percent
Shoot clumps	28	66,7
Bulblets	7	16,7
Hairy roots	1	2,4
Embryos	2	4,8
Calli	3	7,1
Cell cultures	1	2,4

Table SM8. Growth regulators and alkaloids induced when cultured *in vitro*, related to the citations in the literature presented in this review.

Alkaloid Type	Elicitor	Concentration mg L^{-1}	Citations	% total
GAL, LYC, crinane and tazzetine types, norgalanthamine, tyramine, narciclasine, haemanthamine, pancracine and homolycorine types	BA	2,0	10,0	12,8
GAL, LYC, crinane and tazzetine types, norgalanthamine, tyramine, narciclasine, haemanthamine, pancracine and homolycorine types	NAA	1,2	7,0	9,0
GAL, D3-galanthamine, crinine and demethylmarithidine, LYC, trisphaeridine, anhydrolycorine and narwedine.	NAA	1,9	6,0	7,7
GAL and n-formyl-norgalanthamine	BA	5,0	5,0	6,4
GAL, LYC and n-formyl-norgalanthamine	2.4-D	1,0	5,0	6,4

GAL, LYC, D3-galanthamine, crinine and demethylmarithidine	BA	0,1	5,0	6,4
GAL, LYC and crinane types	NAA	0,2	4,0	5,1
GAL and LYC	4'-O-methylnorbelladine	100,0	2,0	2,6
GAL and LYC	4'-O-methylnorbelladine	300,0	2,0	2,6
GAL and LYC	BA	3,0	2,0	2,6
GAL and LYC	Picloram	6,0	2,0	2,6
Alkaloids	2.4-D	0,5	1,0	1,3
alkaloids	NAA	3,0	1,0	1,3
Cherylline, crinamidine, crinine, epibuphanisine, LYC, powelline, undulatine, 1-epideacetyl-bowdensine and 3-O-acetylhamayne	Charcoal	not shown	1,0	1,3
GAL	1-aminocyclopropane-1-carboxylic acid (ACC).	not shown	1,0	1,3
GAL	2.4-D	5,5	1,0	1,3
GAL	2.4-D	11,1	1,0	1,3
GAL	Dicamba	11,1	1,0	1,3
GAL	Methyl jasmonate (MJ)	5,6	1,0	1,3
GAL	No GR		1,0	1,3
GAL	Silver thiosulphate (STS)	not shown	1,0	1,3
GAL and LYC	Jasmonic acid		1,0	1,3
GAL and LYC	NAA	0,1	1,0	1,3
GAL and LYC	TDZ		1,0	1,3
GAL and LYC	Tyrosine and phenylalanine	not shown	1,0	1,3
GAL, LYC and crinane types	2.4-D	0,2	1,0	1,3
GAL, LYC and crinane types	Kinetin	2,0	1,0	1,3
GAL, LYC and others	KMnO4	4500,0	1,0	1,3
GAL, LYC, haemanthamine and homolycorine type	Paclobutrazol	2,9	1,0	1,3
GAL, LYC, haemanthamine and homolycorine type alkaloids.	IBA	not shown	1,0	1,3
GAL, LYC, haemanthamine and homolycorine type.	BA	0,5	1,0	1,3
GAL, trisphaeridine, anhydrolycorine, crinine, demethylmaritidine and narwedine.	Picloram	2,4	1,0	1,3

Ismine, trisphaeridine, GAL , sanguinine, vitattine, 8-O-demethylmaritidine, anhydrolycorine, 11,12-dehydroanhydro-lycorine, montanine, pancracine, 11-hydroxyvitattine, LYC, pseudolycorine.	Methyl jasmonate (MJ)	4,0	1,0	1,3
LYC	2.4-D	2,0	1,0	1,3
LYC	4'-O-methylnorbelladin e	200,0	1,0	1,3
LYC	BA	1,0	1,0	1,3
Total alkaloids	BA	1,5	1,0	1,3
Total alkaloids	*Fusarium sporotrichioides*	not shown	1,0	1,3
trisphaeridine, tazettine, and 11-hydroxyvittatine	Picloram	12,1	1,0	1,3
GAL	Trans-cinnamic acid	1000,0	1,0	0,0

Table SM9. Bioreactors and immersion systems employed for Amaryllidaceae alkaloids in *in vitro* cultures according with the literature reviewed.

Culture system	Observations	Citations	Percent
LSM (liquid shake medium)	110 rpm	5	27,8
Temporary immersion	Bioreactor Rita ®	4	22,2
Temporary immersion system1 L bioreactor vessel		3	16,7
LSM (liquid shake medium)	120 rpm	2	11,1
Bubble column bioreactor		1	5,6
LM	Partially submerged	1	5,6
LSM (liquid shake medium)	100 rpm	1	5,6
LSM (liquid shake medium)	150 rpm	1	5,6

CAPÍTULO II

Validation and quantification of the alkaloid montanine in wild plants and aseptic cultures o*f Rhodophiala bifida*

Introduction

The genus *Rhodophiala* (Amaryllidaceae) is native from South America and comprises more than 30 bulbous species that show ornamental potential because of their colorful and attractive flowers, usually grow in restricted locations, geographically isolated and, like other Amaryllidaceae, have a very slow vegetative multiplication (Muñoz *et al*., 2009). The alkaloids class in this family has been extensively studied because of the galantamine anticholinesterase activities in the treatment of Alzheimer's disease, the cytotoxicity and antitumor action of licorine, and recently, antimicrobial and immunomodulatory activities of montanine (J. P. de ; Andrade *et al*., 2015; Castilhos *et al*., 2007; Harvey, 1995; Oliveira *et al*., 2014; Rhee *et al*., 2001).

The *Rhodophiala bifida* (Herb.) Traub is a native American species of occurrence in South of Brazil and Argentine, which has montanine the alkaloid as the molecule of greatest abundance in its composition (J. P. de ; Andrade *et al*., 2015; Castilhos *et al*., 2007; Oliveira *et al*., 2014), as verified by different analytical methods to identify and quantify Amaryllidaceae alkaloids in plants (Bastida *et al*., 2011; Kaya *et al*., 2010; Yubin *et al*., 2014).

In vitro cultivation is an essential tool in metabolite production mainly for plants that have a slow growth cycle such as *R. bifida*. This technique allows the plant to be produced in the laboratory, without seasonal variations, pests, diseases and inconsistencies in the quality of the products or the crop, under specific and controlled growth conditions, clean culture and the possibility of a calculation of future yield of the molecule to be obtained. In addition it was avoid the overharvesting of the natural stands (Taleb *et al*., 2014). *In vitro* regeneration and micropropagation of *R. bifida* is the first step in the production of the alkaloids present in this plant through biotechnology. The aim of the present work was to develop and validate an analytical method using HPLC-PDA for the quantification of the alkaloid present in *Rhodophiala bifida* plants and to quantify this alkaloid in wild, *in vitro* grown and acclimatized plants.

Experimental

Chemical

Inositol, nicotinic acid, thiamine, pyridoxine, glycine, agar, activated charcoal, sucrose, naphthalene acetic acid (NAA), 6-benzylaminopurine (BA), casein and phytagel (Sigma-Aldrich, Germany), acetonitrile and methanol HPLC grade (J. T. Baker Chemicals, EUA); ethyl ether, ethyl acetate, anhydrous sodium sulfate and sulfuric acid 36% analytical grade (Synth, São Paulo), ammonium hydroxide 25% analytical grade (Merck, Germany). Ultra-pure Milli-Q water (Millipore ®), PVDF sample filtration systems 0.45 μm for HPLC and 0.22 μm for UPLC-MS (Millipore ®).

Plant material

The plant material used in the procedures was collected in Pelotas (RS) (Latitude: -31.7719; Longitude: -52.3425; err: ± 51462 WGS84). The botanical identification was performed by Dutilh J. H. A. and a complete individual was deposited in the Herbarium of the Natural Sciences Institute of the Federal University of Rio Grande do Sul - UFRGS, under the identification ICN 192333 (Zuanazzi, 2013).

R. bifida plants were harvested in the years of 2013 (March), 2014 (March) and 2015 (July), the bulbs were separated and washed in running water, dried in a stove at 40 ºC until constant weight, followed by grinding with knife mills and receiving code WP13, WP14 and WP15, respectively.

Part of the bulbs collected in 2014, after washing under running water, underwent a pre-disinfestation process in 2% sodium hypochlorite for 3 hours, washing in distilled and autoclaved water and oven drying at 23ºC for 24h. After this period, the bulbs were stored at room temperature for 15 days. These samples were destined to *in vitro* culture and received the IC (*in vitro* cultures) code.

In vitro cultivation and acclimatization of *R. bifida* plants

The IC bulbs had their outer, darker layers removed and underwent the disinfestation process which consisted of: (i) shaking in 70% alcohol solution for 1 minute, (ii) agitation in 8% sodium hypochlorite plus tween 20 (2 drops for 100 mL) for 30 minutes and (iii) washing in distilled and autoclaved water (3 times). In a laminar flow chamber, the bulbs were dried on sterile filter paper, sectioned into 1.0 x 1.5 cm fragments and inoculated into Petri dishes containing 20 mL of modified MS medium (Kyte *et al.*, 2013).

In order to induce shoots in the bulbous explants of *R. bifida*, inoculation was carried out in medium containing the macronutrients of the MS medium (Murashige and Skoog, 1962),

inositol 100 mg L^{-1}, nicotinic acid 0.5 mg L^{-1}, thiamine 0.1 mg L^{-1}, pyridoxine 0.5 mg L^{-1}, casein 1 g L^{-1}, glycine 2 mg L^{-1}, phytagel 2 g L^{-1}, sucrose 30 g L^{-1}, pH 5.5 and autoclaved at 121 °C at 1 atm for 20 min. In addition, growth regulators were sterilized by membrane filtration (Millipore® 22 μm) and added to the medium after autoclaving, naphthalene acetic acid (NAA) 0.1 mg L^{-1} and 6-benzylaminopurine (BA) 0.5 mg L^{-1} according to protocol previously established (data not shown).

After the shoot growth, they were transferred to flasks containing culture medium for multiplication. This medium consisted in MS medium base with the addition of inositol 100 mg L^{-1}, nicotinic acid 0.5 mg L^{-1}, thiamine 0.5 mg L^{-1}, pyridoxine 0.5 mg L^{-1}, glycine 1 mg L^{-1}, agar 7 g L^{-1}, activated charcoal 5 g L^{-1}, sucrose 30 g L^{-1}, pH 5.5. After the multiplication of the shoots, the plants were transferred to liquid MS medium like before, without the charcoal and gellying agent. The autoclaving process was as described in the first culture medium.

The media containing the microplants were renewed every 4 weeks. The harvest occurred when the plants completed 30, 60 and 90 days of growth. The microplants were oven dried at ± 40 °C and ground to be chemically evaluated.

After 90 days, some plants were acclimatized, removed from the *in vitro* culture and placed in autoclaved soil, composed of vermiculite and soil (50:50; w/w). During the acclimatization period, they were left in a room with natural light and irrigated daily for 60 days, when they were collected, dried in an oven at ± 40 ° C and ground to be chemically analyzed.

Sample preparation

Method validation

For the method validation, a sample was prepared using 1g of *R. bifida* bulbs from WP14. The extraction of total alkaloids was performed according to the proposed technique (Georgieva *et al*., 2007) with modifications, using 2% sulfuric acid (V/V) on ultrasound bath for 4 hours. The samples were filtered and the supernatant washed with ethyl ether (3 x 100 mL), the aqueous fraction was basified with 25% ammonium hydroxide (V/V) until pH 9. This basic aqueous solution was partitioned with ethyl acetate (3 x 100 mL). The organic residue was filtered over anhydrous sodium sulfate and the volume was reduced to the residue in a rotatory evaporator. The alkaloid rich residue was subjected to vacuum liquid chromatography (VLC) with silica gel using 300 mL of ethyl ether first, and then 500 mL of methanol, collecting

the fractions separately. The methanol fraction was taken to the residue in the rotatory evaporator at 40 °C, resuspended in ultrapure water, frozen and lyophilized. After lyophilization, a stock solution of 1 mg mL^{-1} in methanol was prepared and stored under refrigeration until use.

Quantification in wild plants (WP) and in vitro cultures (IC)

Samples of wild and *in vitro* cultured plants were evaluated for the presence of montanine-type alkaloids as well as the concentration of montanine in the total alkaloid extract. To prepare the samples in triplicate, 90 mg of dry and micronized WP and IC material were used and the entire extraction process was performed as previously described, omitting the VLC procedure. Therefore, after filtration of the ethyl acetate fraction, this was taken to the residue and the contents resuspended in 1 ml of methanol and filtered in 0.45 μm membrane for further HPLC analysis.

Chromatographic conditions

Chromatographic analyzes were performed on a Waters Alliance e2695 with a diode arrangement detector (PDAWaters 2998), and software Empower 3 HPLC (Waters®) for data acquisition and treatment. The chromatographic column employed was reverse phase (Synergi Polar Phenomenex, 80 Å, 4 μm, 250 x 4.60 mm). The elution system used was composed by mobile phase A with water: trifluoroacetic acid (TFA) (100: 0.01 %, V/V) and mobile phase B, acetonitrile: TFA (100: 0.08 %, V/V). The elution mode selected was isocratic containing 30% of the mobile phase B for a period of 10 minutes and flow rate of 0.7 mL min^{-1}, column temperature at 25 ℃ and the sample compartment at 20 ℃. Scanning of the chromatographic injections in the range of 200-400 nm was performed and the wavelength of 254 nm was selected as the most suitable.

Montanine characterization in UPLC-MS

The characterization of the montanine used for validation of the methodology was carried out using UPLC-MS (Waters Q-TOF Premier), with electrospray ionization (ESI) in positive mode, with a voltage of 3.0 kV in capillary, nitrogen as desolvation gas at 250 °C, flow rate of 350 L/h, and 30 eV of impact energy to obtain the mass spectrum. The results obtained were duly confronted with data available in the literature (Brown, 1968; Oliveira *et al*., 2014; Ulubelen, 1990; Wildman and Olesen, 1976).

Validation by HPLC-PDA

The validation of the present study was performed according to Resolution n. 899 of 2003 (ANVISA, 2003) and the ICH (ICH, 2005), with the following parameters being evaluated.

Peak purity test

The peak purity test was performed by observing impurities through cuts at different parts of the chromatographic peak.

Linearity

For the linearity analysis, six solutions containing montanine were prepared at concentrations of 0.01 to 0.5 mg mL^{-1} to be analyzed in triplicate, on three different days.

Precision (Repeatability and Intermediate precision)

Intra-analysis precision (repeatability) was established by injections containing three concentrations of the solution with 0.1mM; 0.05 and 0.02 mg mL^{-1} (low, medium and high), prepared in triplicate. Intermediate or inter-analysis precision was determined by three injections of the solution containing montanine at 0.05 mg mL^{-1} on three different days.

Limit of Detection (LOD)and Limit of Quantification (LOQ)

The LOD and LOQ calculations were performed using equations A and B from baseline noise, as follows:

$$(A)LOD = \frac{(SDa\ x\ 3)}{CC}$$

$$(B)LOQ = \frac{(SD\ x\ 10)}{CC}$$

Where: SDa is the standard deviation of the intercept with the Y axis of analytical curves, is performed in triplicate containing concentrations close to the assumed LOQ. SD is the standard deviation of samples from white and CC is the slope of the calibration curve.

Accuracy

The accuracy of the method was verified by the addition of concentrations of solution containing montanine at three known concentrations (0.02, 0.05 and 0.1 mg mL^{-1}).

Robustness

The susceptibility of the method to variations was evaluated from the injection of solutions containing montanine in a concentration of 0.05 mg mL^{-1}. The modified parameters of the analytical method are represented in the Table 1.

Table 1. Parameters changed to evaluate robustness for the proposed method.

Parameters	**Proposed method**	**Modified**
Mobile phase	Mobile phase B (ACN: TFA 100: 0.08 %, V/V) at 30%	Mobile phase B at 35%
		Mobile phase B at 25%
Chromatografic column	Synergi Polar RP (250 x 4.6 mm; 4 µm)	Novapak C18 (250 x 3.9 mm; 4 µm)
Column temperature	25 ℃	35 ℃
		45 ℃
Flux	0.7 mL min^{-1}	0.6 mL min^{-1}
		0.8 mL min^{-1}
Acid in the mobile phase	Mobile phase A (Water + TFA 0.01%)	Mobile phase A: Water + TFA 0.02%
		Mobile phase A: Water + TFA 0.05%
Different equipment	Waters Alliance e2695 and diode array detector (Waters PDA 2998)	Waters Alliance 2695 and diode array detector (Waters PDA 996)

Montanine: identification and quantification in *Rhodophiala bifida* wild, *in vitro* and acclimatized plants

The wild samples correspond to plants WP13, WP14 and WP15 and samples cultured according to the protocols previously described, cultivated in liquid medium and solid medium, for 60 and 90 days (IC-LM 30 days, IC-LM 60 days, IC-SM 60 days, IC-LM 90 days and IC-SM 90 days), the extraction and analyzing process occurred as above.

Statistical analysis

All experiments related to chemical analyzes were performed using three biological replicates, three times with three technical replicates for each sample. Data were analyzed using the Winstat® program. Analysis of variance (ANOVA) and means tests were compared using the Tukey test, with a 5% level of significance (P <0.05).

Results and discussion

Montanine: characterization and development of chromatographic validation method

Validation was conducted on a previously established protocol aiming at determinating a specific analyte in varied concentrations one according to regulatory requirements in pharmaceutical analysis, as well as providing documentary evidence and a high degree of certainty that the analytical method employed for a specific test is appropriate for that purpose (Shabir, 2004; Thompson *et al*., 2002).

Currently, there is little information on phytochemical analysis or standardization of montanine (Fig. 1) or even *R. bifida* extracts. Thus, we sought to develop and validate a method of analysis and quantification of montanine in plants of *R. bifida* using HPLC-PDA. The chromatographic analysis was performed at a wavelength of 254 nm.

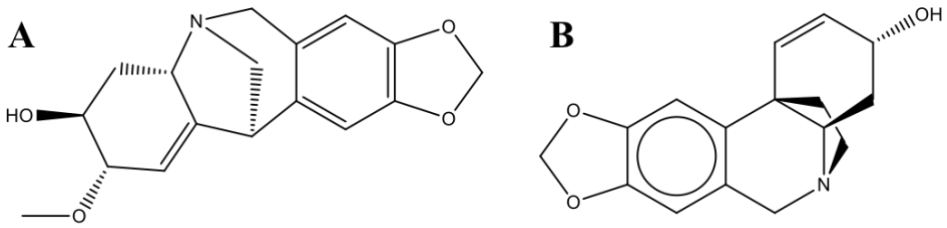

Fig. 1. Molecular structure of alkaloids: (**A**) montanine; (**B**) crinine.

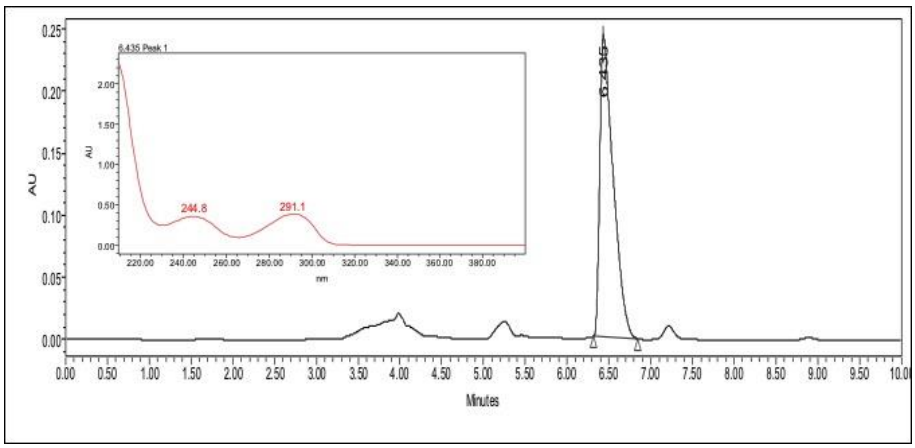

Fig. 2. Chromatogram showing montanine extracted from *Rhodophiala bifida* plants, using reverse phase column (Synergi Polar RP, 80 Å, 250 x 4.60 mm) and wavelength of 254 nm.

As shown in the chromatogram of Fig. 2, the peak with retention time close to 6.4 minutes was analyzed for its UV $\lambda_{máx}$ (Fig. 3) and MS/MS fragmentation. According to the spectrum in the ultraviolet (UV) region found in the HPLC analyzes, it was possible to observe that the compound exhibits maximum absorptions at 244 and 291 nm, characteristic of common chromophore between the montanine-type alkaloids found in Amaryllidaceae.

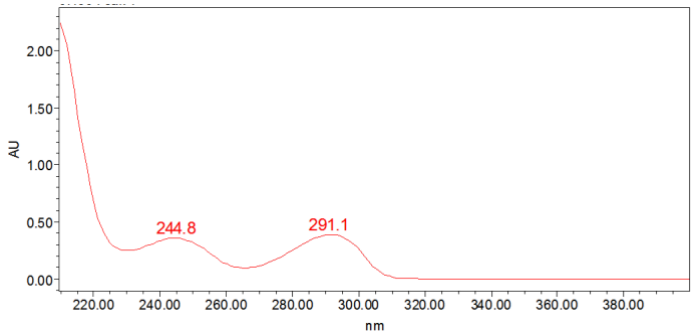

Fig. 3. Spectrum in the ultraviolet region of the solution containing montanine extracted from *Rhodophiala bifida.*

The analysis of the fragmentation profile of the mass spectrum obtained (Fig. 4) showed the protonated molecular ion $[M+H]^+$ in *m/z* 302.1456 (calculated 301.1456 for the majoritary peak in the alkaloid extraction from *R. bifida*). The fragments of the molecule (*m/z* 270.1213; *m/z* 252.1108; *m/z* 223.0833; *m/z* 185.0674; *m/z* 174.0616; *m/z* 165.0753; *m/z* 135.0496 e *m/z* 79.0599, as depicted in Fig. 4.

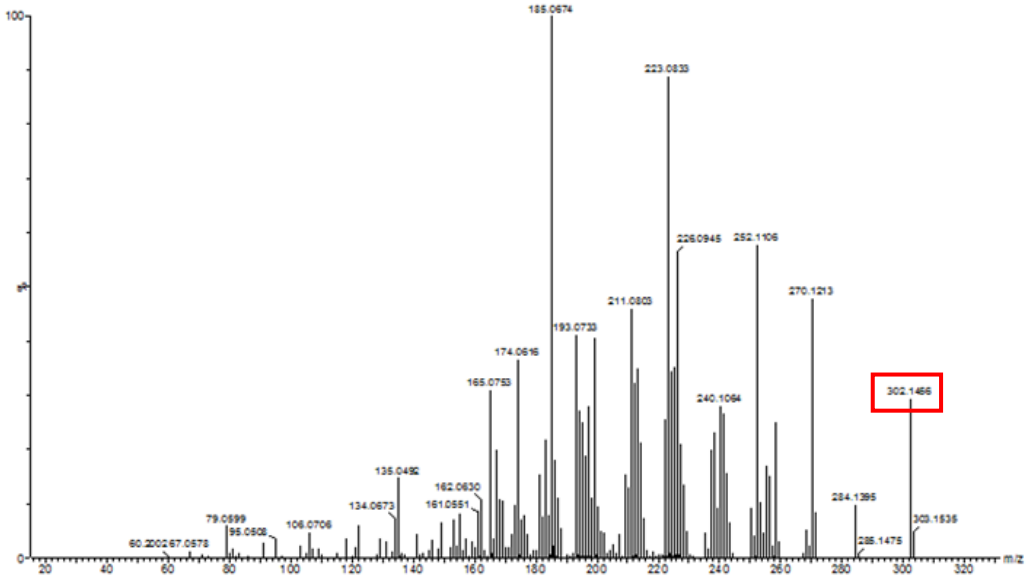

Fig. 4. Mass spectrum of the montanine molecule from the total alkaloids extracted from *Rhodophiala bifida* bulbs.

According to the identified molecular losses, the losses of a methoxyl followed by the loss of a water molecule could be observed; also the loss of the fragment CH_2=NH can be mentioned. Previously reported analyzes (Kinstle *et al*., 1966; Silva, 2005) indicated that the loss of the fragment of CH_2=NH for the generation of the fragment *m/z* 223 it is found only for alkaloids montanine and coccinine. Therefore, other compounds that present the same molecular weight, such as α and β-isocrinamine can be discarded based on this hypothesis of fragmentation profile. The presence of the ion *m/z* 174 in the spectrum confirms the presence of the ring system of the montanine-type alkaloids (Wildman and Brown, 1968). With this information, the suggested molecular formula for montanine alkaloid is $C_{17}H_{19}NO_4$, (Fig. 1*A*).

Peak purity

The purity of the chromatographic peaks of the montanine were evaluated using a PDA detector. As can be seen in Fig. 5*A*, *B* and *C*, the wavelengths of the ultraviolet spectrum are the same over the entire peak under analysis, demonstrating that it is attributed to a single component.

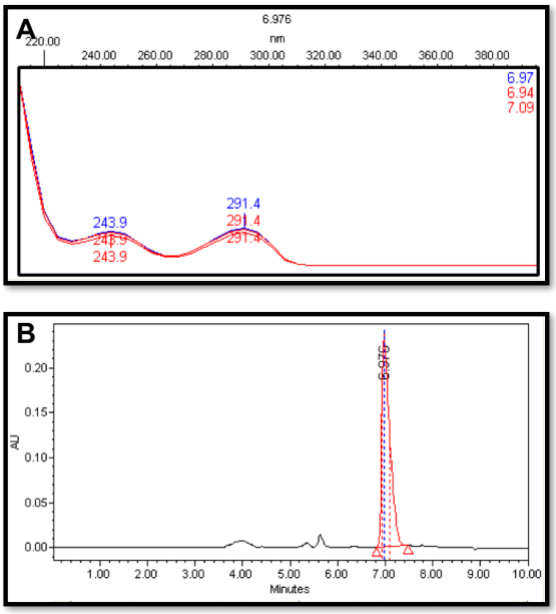

Fig. 5. Chromatogram demonstrating purity of the chromatographic peak of montanine extracted from *Rhodophiala bifida* bulbs. (A) Ultraviolet spectrum equivalent to the chromatographic peak cuts; (B) Region of cut selected for analysis.

1.1.1 Linearity

The evaluation of the linearity of the method was performed by constructing three linearity curves on three different days with the solution containing montanine in six different concentrations in the range of 0.01 to 0.5 mg mL^{-1}. Linearity curves showed good linear correlation, with R^2 = 0,99963, with linear equation of $y = 5206{,}9x + 5848{,}5$, as can be visualized in Fig. 6.

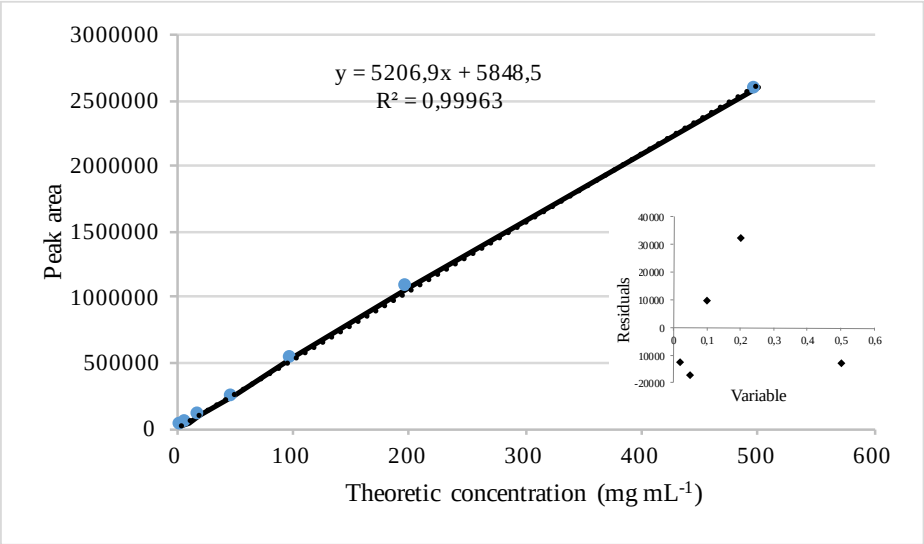

Fig 6. Linearity curve and plot of the residues for the solution of montanine.

Repeatability and accuracy

The precision parameter was evaluated by performing the intra-day (same day) variability test (containing 3 concentrations in triplicate), where the relative standard deviation (RSD) was 3.4%, and by observing the variability of the precision in three consecutive days (analysis of three samples, injected in triplicate), where the RSD of 0.165% was observed (Table 2). The RSD values found for both tests are below the limits recommended in the analytical guides, showing to be accurate and reproducible.

Table 2. Relative standard deviation (RSD) to the peak areas found in the evaluations of precision and robustness.

Precision (montanine 0.05 mg mL^{-1})	Peak area ± RSD	Retention time (min)
Intra-days (Same days)	307730.39 ± 3.40	6.57
Inter-days (Different days)	263834.7 ± 0.16	6.58
Robustness		
Changes in mobile phase ratio		
65:35 (%)	258500 ± 2.98	5.65
70:30 (%)	247899 ± 0.12	6.58
75:25 (%)	251442 ± 0.27	8.60
Mobile phase flux		
0,6 mL min^{-1}	288108 ± 0.66	7.64
0,7 mL min^{-1}	247899 ± 0.12	6.58
0,8 mL min^{-1}	216421 ± 0.25	5.78
Column temperature		
25°C	249978 ± 0.12	6.58
35°C	253420 ± 0.14	6.34
45°C	255874 ± 0.97	6.20

Different pH in mobile phase		
Water + TFA 0,01% - pH 4.04	271540 ± 0.07	6.60
Water + TFA 0,02% - pH 3.67	265481 ± 3.25	6.56
Water + TFA 0,05% - pH 3.27	276543 ± 1.58	6.52
Mobile phase preparation		
Mode 1- Fase A and B united by HPLC	271540 ± 0.07	6.60
Mode 2 - Fase B poured in fase A	281165 ± 0.64	7.09
Mode 3 - Fase A poured in fase B	284599 ± 0.40	9.90
Different machine		
Waters Alliance e2695	274835 ± 2.21	6.67
Waters Alliance 2695	251922 ± 1.15	6.93

Abbreviations: TFA (trifluoroacetic acid).

LOD and LOQ

The evaluation of the limits of detection and quantification are determined with the purpose of defining the sensitivity of the method by means of equations. Thus, LOD is defined as the lowest concentration of analyte that can be detected in a sample with 1% chance of finding a false positive and LOQ is the lowest content of an analyte that can be detected in a sample with the chance of a false negative in 5% of the analyzes. By analyzing samples with a known concentration of montanine, a detection limit of 0.0067 μg mL^{-1} and a limit of quantification of 0.0225 μg mL^{-1} were found, with the points of the curve being above, since the method proved to be linear in a concentration of 0.01 mg mL^{-1}.

Accuracy

The accuracy of the method was performed analyzing samples of alkaloid rich extract from *Rhodophiala bifida* plants to which known concentrations of montanine solution were added. Nine determinations were performed at three different concentrations, 0.02 mg mL^{-1} (low); 0.05 mg mL^{-1} (average) and 0.1 mg mL^{-1} (high), at the levels of 40% to 200% of the central concentration, containing 3 solutions each. The results are shown in Table 3, and all solutions showed high recovery rates and were reproducible at all concentrations evaluated.

Table 3. Recovery of montanine in samples of *Rhodophiala bifida*.

Sample	Concentration		Recovery (%)	Average (%)	Average overall (%)
	Teorical (mg mL^{-1})	Obtained (mg mL^{-1})			
Low (0.02 mg mL^{-1})	0.7493	0.7287	97.2		
	0.7321	0.7316	99.9	98.9	
	0.7311	0.7272	99.5		

Average (0.05 mg mL^{-1})	0.7447	0.7578	101.8		
	0.7553	0.7600	100.6	101.5	100.6
	0.7466	0.7613	102.0		
High (0.1 mg mL^{-1})	0.7622	0.7806	102.4		
	0.7622	0.7680	100.8	101.5	
	0.7703	0.7795	101.2		

Robustness

Robustness of a method is its ability for an analytical procedure to remain unchanged against small changes in its parameters. This analysis was evaluated by modifications in the ratio of the mobile phase (MP), MP flow, column temperature, acid addition to MP (or pH) and different apparatus, data shown in Table 2. These changes did not demonstrate significant changes in peak area and retention time, demonstrating that the method is specific, accurate and precise.

Quantification analysis

The methodology developed in this study presents a short analysis time because, when dealing with complex samples such as plant extracts, it presents the major peak in the retention time of about 8 minutes, turns out to result in a small mobile phase consumption and lower total cost of analysis. Thus, the validated technique was used to quantify the concentration of montanine present in wild *Rhodophiala bifida* plants in three distinct years, cultivated *in vitro* in two types of culture medium (semisolid and liquid) and three different acclimatized periods.

Table 4. Amount of montanine from *Rhodophiala bifida* from different types of culture and analised by HPLC-PDA.

Samples	**Montanine (mg g^{-1} dry plant)**	**SD**
WP - 2013	2.114 A	± 0.245
WP - 2014	1.994 A	± 0.146
WP - 2015	1.374 B	± 0.119
IC-LM 30 days	0.307 D	± 0.013
IC-LM 60 days	0.296 D	± 0.065
IC-LM 90 days	0.204 D	± 0.046
IC-SM 60 days	0.230 D	± 0.026
IC-SM 90 days	1.051 C	± 0.021
Aclimatized	0,294 D	± 0.004

Means with different letters are significantly different using Tukey test ($P< 0.05$). Abbreviations: WP - Wild plants; IC - *in vitro* culture; LM - liquid medium; SM - solid medium; SD - standard deviation.

Montanine concentration of the samples were calculated by means of the linear equation $y = 5206.9x + 5848.5$ and statistical analysis. As can be seen in Table 4, the most significant values in the montanine content were found in samples of wild plants collected in 2013 and 2014, with 2.11 and 1.99 mg of montanine in each gram of dry plant, the content of the plants collected in the year 2015 present good results too, 1.37 mg g^{-1} and the plants grown *in vitro*, after 90 days (IC-SM 90 days) of culture in solid medium had the fourth highest amount (1.05 mg g^{-1} dry plant). Different from our results, studies with the *Hymenocallis littoralis* plant, comparing the levels of licorine in wild plants and callus cultures, have reported that some callus lines presented higher content of licorine when compared to wild plants (Subramaniam *et al*., 2014).

According to data collected in the harvest in Pelotas region (Table 5), it can be observed that mean annual temperatures differed between the months of harvest of wild plants, demonstrating that in the month which happened the crop of the year of 2015, the averages of air and soil temperatures were much lower, rainfall and relative humidity were higher, there was a lower incidence of solar energy and higher wind speed, suggesting that in situations where the plant would be entering its period of dormancy, near the winter, the plants of *R. bifida* showed a decrease in the biosynthesis of montanine.

Studies with *Zephyranthes grandiflora* have shown that the content of AmAl (Amaryllidaceae Alkaloids) may differ between different species, seasons or stages of plant development, with each alkaloid following a pattern, some having their highest concentrations in rainy seasons during flowering, and lower productivity during the winter, which would be the dormancy stage of the plant, like what we found in our study (Katoch *et al*., 2012).

Evaluations conducted with plants of *Crinum macowanii* (Elgorashi *et al*., 2002) found wide variations in the content of alkaloids during the seasons, as shown with *Z. grandiflora*, and some alkaloids like crinine (Fig. 1*B*) show an increase during the winter, unlike what was observed in the montanine samples studied by us. The time of yield of herbal drugs is an important point, because of its variation during the year, surveys report that there are seasonal variations in the contents of almost all classes of secondary metabolites, among them we can include the alkaloids. For annual plants, seasonal studies may be confused with metabolic changes in the plants own development process, controlled by hormones, and should be considered together. Germination, plant elongation, bud growth and flowering, processes that

undergo a great hormonal influence, for example changes in metabolic profile and expected; in the case of *R. bifida*, in the months in which the first two collections took place, the plants had flowers and some seeds, whereas in the collection of the year 2015, there were only the leaves in the aerial part of the plant (Gobbo-neto and Lopes, 2007; Pavarini *et al*., 2012).

Table 5. Monthly climatological report of the Automatic Weather Station* of Embrapa Clima Temperado de Pelotas - RS, in the months of collection of wild *Rhodophiala bifida* analyzed.

Month/Year of harvest		**Air temperature (°C)**			**Soil temperature at 5 cm (°C)**	**Rainfall (mm)**	**Relative humidity (%)**	**Solar energy (cal.cm^{-2}.day^{-1})**	**Wind velocity (Km/h)**	
		Avrge	**Max**	**Min**					**Avrge**	**Max**
March 2013	Avrge	20.3	26.0	15.9	23.3	1.8	77.8	399.9	2.7	22.4
	Max	25.7	31.8	22.0	29.9	27.7	92.2	560.4	6.4	33.8
	Min	16.4	21.8	11.1	19.4	0.0	59.0	99.4	0.0	12.9
March 2014	Avrge	21.3	26.6	17.5	23.7	5.6	77.5	393.5	4	24.0
	Max	24.5	32.2	20.7	27.6	46.0	94.2	583.4	10.7	46.7
	Min	16.0	19.3	11.8	19.1	0.0	58.0	0.0	0.0	0.0
June 2015	Avrge	14.3	19.4	9.9	14.1	7.0	83.7	209.1	6.0	25.4
	Max	22.6	26.9	18.7	19.6	50.3	97.5	309.9	13.0	46.7
	Min	7.3	13.5	3.3	9.0	0.0	56.8	27.2	2.1	12.9

*Altitude at 57m. Abbreviations: Avrge (average); Max (maximum); Min (minimum).

Observations can also be made regarding temperatures changes. Findings from the literature indicate that there are increases in some essential enzymes for routes such as PAL (phenylalanine ammonia-lyase) and chalcone synthase, as well alkaloid in some species such as tobacco (*Nicotiana tabacum*), generating an increase in the biosynthesis of secondary metabolites (SM) (Gobbo-neto and Lopes, 2007). These reports are in agreement with our results, in which the air and soil temperatures were higher in the months suggesting that this contributed to plants having a higher concentration of montanine. In relation to water stress, it is known that it produces they produce significant consequences in the concentrations of SM, often increasing the alkaloids, and that in cases of continuous rains they can cause leaching of water-soluble compounds from the leaves and roots, resulting in loss of these molecules, this may have influenced our data, in which increased rainfall and relative humidity was associed a decrease in production of the alkaloid under study. As for solar energy, in plants such as *Atropa belladonna*, *Datura stramonium* and *Cinchona ledgeriana*, greater exposure to sunlight leads to an increase in its alkaloid content. In plants of *R. bifida*, greater sunlight coincide with higher production of montanine (Gobbo-neto and Lopes, 2007).

Studies with *in vitro* cultured plants showed a higher concentration of montanine found in plants grown for 90 days in solid medium, and these values are quite expressive when

compared to other conditions and periods of *in vitro* culture. The culture media of all *in vitro* cultured plants showed the same amount of macro and micronutrients, except that the solid media presented agar (7 g L^{-1}) and activated charcoal (5 g L^{-1}) in its composition. In studies to determine the influence of different concentrations of agar and liquid media on the propagation of *Boswellia serrata*, it was observed that the liquid media favored the multiplication of shoots and the rooting of the plants, with an increase in the content of chlorophyll and biomass (Suthar *et al*., 2011). Our results were partially divergent, because the liquid medium was equally efficient in the multiplication of these plants (data not provided), but it was not effective when related to the increment of the alkaloid analyzed.

Another important factor which may have contributed to different results was the addition of activated charcoal which is widely used in tissue culture for increasing cell growth and tissue development. This constituent also presents essential role in the micropropagation, rooting, elongation of shoots and formation of bulbs. It is believed that the promoter effects of charcoal are due to the irreversible adsorption of molecules that can be toxic to plant tissues, such as phenolic compounds, allowing a better development of the plant. Also, this molecule helps to promote soil-like conditions by causing a darkening of the culture medium; decreases the hyperhydricity conditions in the plant tissue and promotes the adsorption of vitamins, metal ions and plant growth regulators (PGR), allowing the latter to be gradually released to tissues (Chandra *et al*., 2013; García-Gonzáles *et al*., 2010; Rice *et al*., 2011).

Some studies (Constabel *et al*., 1974; Rao and Ravishankar, 2002; Rueffer, 1985) report that the addition of adsorbent substances such as activated charcoal, cause adsorption *in situ* and may increase the production of SM, as found in reports of studies with cell cultures of plants such as *Matricaria chamomilla*, *Nicotiana tabacum* and *Vanilla fragrans* where there was an increase in the biosynthesis of the metabolites under study, due to the stimulation of the release of these molecules in the medium.

Conclusions

Changes in the montanine concentration of *Rhodophiala bifida* plants are the initial step in the processes concerning the production of these plants in the field, *in vitro* or the acclimatization to growth these selected varieties in the green house or field. Through these studies, we developed a reliable, specific and robust chromatographic method for the quantification of montanine in samples of *R. bifida*. Moreover, we detect that the cultivation under summer temperatures, lower rainfall and humidity can increase the productivity of

montanine in the field. *In vitro*, plants after 3 months present better production of montanine, when cultivated in solid medium.

Due to the fact that this alkaloid has several biological activities (J. P. de ; P. de ; Andrade *et al*., 2015; Andrade, 2007; Castilhos *et al*., 2007; da Silva *et al*., 2006; De Andrade *et al*., 2012; De Brum Vieira *et al*., 2011; Giordani, 2010; Oliveira *et al*., 2014; Ribeiro *et al*., 2009; Silva, 2005) and a patent on some. Its application was recently licensed by our research group (Oliveira *et al*., 2014) the results obtained with *in vitro* cultures may be a tool in future studies with this plant, including biotechnological approaches seeking to increase production of this alkaloid, as well as facilitate studies to on definine biosynthetic routes of montanine.

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CAPÍTULO III

*Characterization of alkaloid production in Rhodophiala bifida (*Amaryllidaceae*) plants*

Introduction

Plants and their secondary metabolism represent a rich source of different chemical compounds with biological activities widely used in the production of new drugs (MORISHIGE *et al.*, 2002). Alkaloids are a large group of plant defense secondary metabolites characterized by the presence of a nitrogen atom in the heterocyclic ring. They are represented by a very large diversity of molecules distributed in several plant classes and possess biological activities useful for treatments of human diseases (TAKOS; ROOK, 2013).

Here, we focus on alkaloids produced in a specific family of plants, the *Amaryllidaceae*. These bulbous plants are used as ornamental species and produce promising drugs such as the already commercialized galanthamine (BICKEL *et al.*, 1991; TAKOS; ROOK, 2013).

Despite all the pharmaceutical interest in galanthamine and the search for new drugs derived from the *Amaryllidaceae* alkaloid (AmAl), the biosynthetic pathway is not yet fully characterized. There are still many unknown enzymatic steps, especially in the pathway not related to galanthamine formation, such as the montanine (**15**) pathway. Montanine is an *Amaryllidaceae* alkaloid, characterized by a unique 5,11-methanomorphanthridine skeleton (JIN; ZHONG, 2013). This molecule possesses a range of activities such as anxiolytic, antidepressive and anticonvulsive (SILVA *et al.*, 2006), acetylcholinestarease inhibiting (ANDRADE *et al.*, 2012), anti-rheumatic (FARINON *et al.*, 2017), antimicrobial (CASTILHOS *et al.*, 2007) and antiproliferative activities (REIS *et al.*, 2008).

The biosynthesis of these alkaloids (Figure 1) begins with the L-phenylalanine (**1**). Phenylalanine ammonia-lyase (PAL) enzyme generates *trans*-cinnamic acid (**2**). CYP73A1cytochrome P450 enzyme then produces *p*-coumaric acid (**3**) which through the action of the CYP98A3 forms 4-hydroxycinnamic acid (caffeic acid) (**4**) or 4-hydroxybenzaldehyde (**5**). Both molecules can generate protocatechuic aldehyde (**6**).

Caffeic acid is transformed by the action of *Vp*VAN paralogue (KILGORE; KUTCHAN, 2015; KORNIENKO; EVIDENTE, 2008; SINGH; DESGAGNÉ-PENIX, 2014).

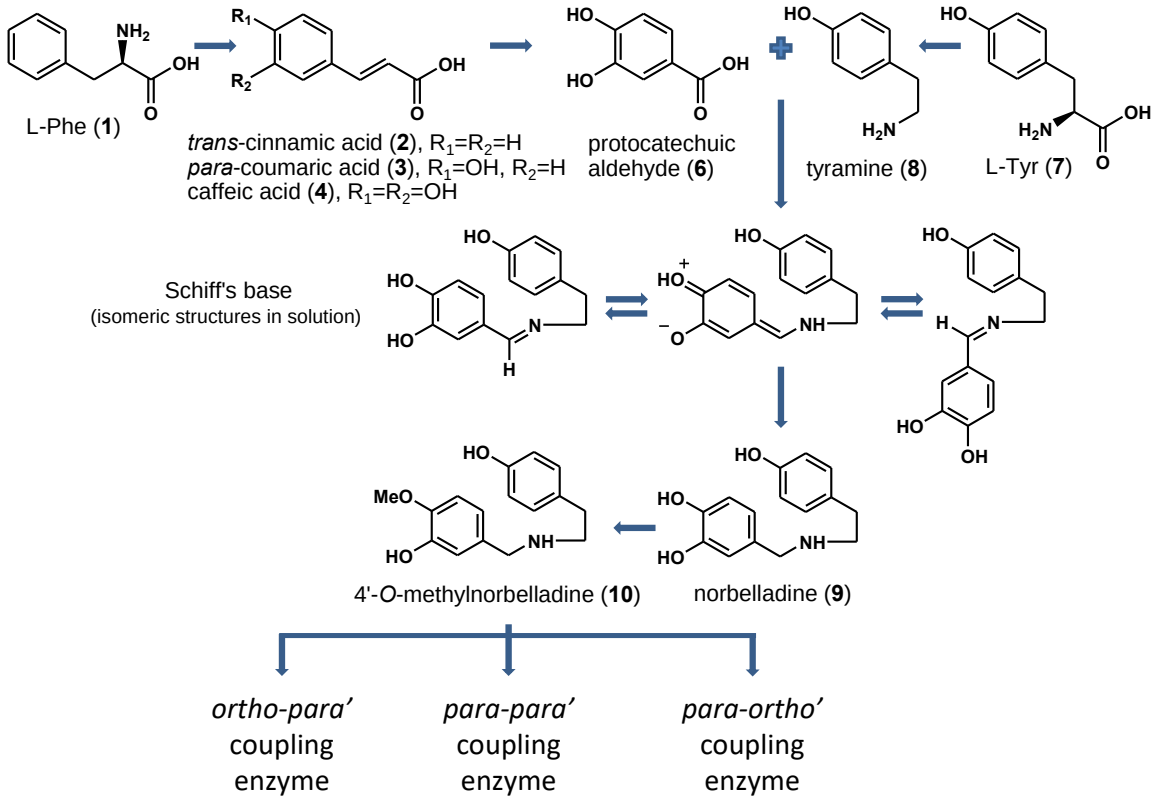

Figure 1. Biosynthetic pathway of the 4'-*O*-methylnorbelladine (10), the Amaryllidaceae alkaloids precursor (KILGORE; KUTCHAN, 2015).

Using L-tyrosine (**7**) tyrosine decarboxylase synthesizes tyramine (**8**). Tyramine can be then condensed to protocatechuic aldehyde (**6**) by an unknown enzyme to a Schiff-base and reduces it by the hypothetical Noroxomaritidine Reductase to norbelladine (**9**). Norbelladine (**9**) is then methylated by norbelladine 4'-*O*-methyltransferase (N4OMT) to form 4'-*O*-methylnorbelladine (**10**) (KILGORE; KUTCHAN, 2015).

The key intermediate for the multiple biosynthetic pathways producing the various structural types of AmAl (Fig. 2) is the cyclization by the CYP96T1 Cytochrome P450 enzyme of 4'-*O*-methylnorbelladine. This cyclization requires phenol-phenol oxidative coupling in three different positions. These are the *ortho-para'*, *para-para'* and *para-ortho'* C-C coupling (EL TAHCHY *et al.*, 2010; KILGORE *et al.*, 2016a; KILGORE; KUTCHAN, 2015; LAURAIN-MATTAR; PTAK, 2016)

The 8-*O*-Demethyloxomaritidine (**11**) is formed following *para-para'* C-C phenol coupling. This compound is a dienone intermediate precursor of the alkaloids

skeleton types such as haemanthamine, tazettine, crinine, narciclasine and montanine. After a reduction of the ketone group, 8-*O*-demethylmaritidine (**12**) is formed and through the formation of an oxide bridge, vittatine (**13**) is produced (EL TAHCHY *et al.*, 2010; KILGORE *et al.*, 2016b; KORNIENKO; EVIDENTE, 2008).

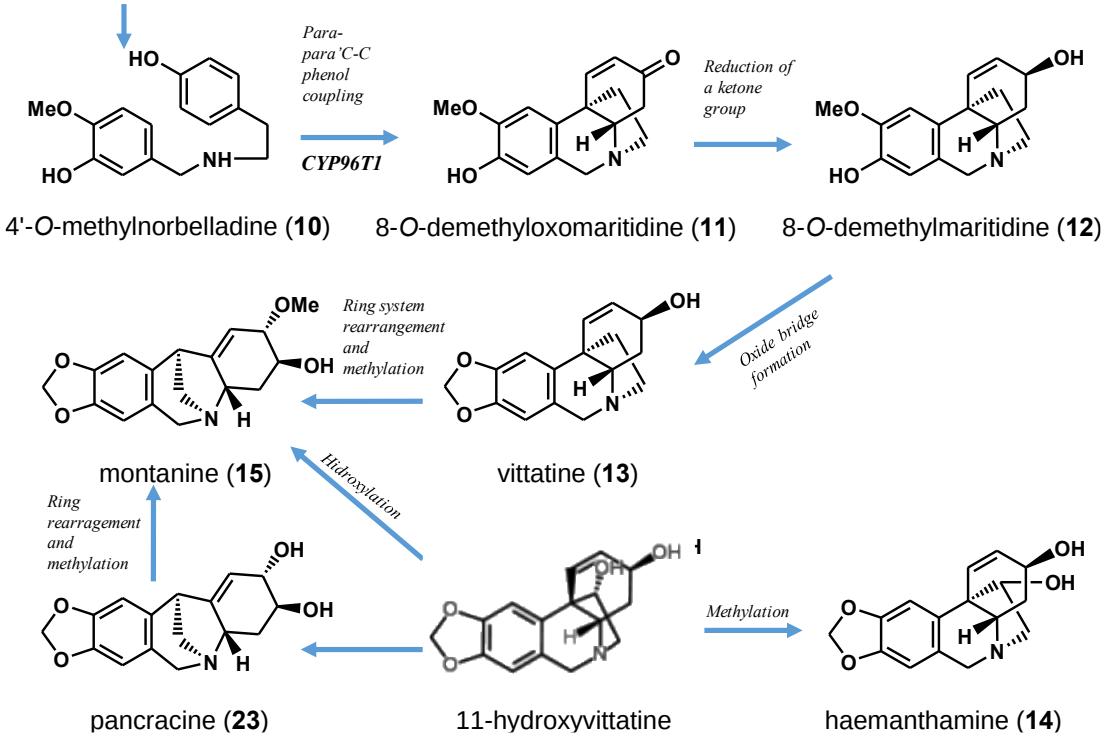

Figure 2. Schematic representation showing the important steps of Montanine types alkaloids putative formation in Amaryllidaceae plants (BASTIDA *et al.*, 2011; KILGORE *et al.*, 2016b; OLESEN, 1976; WILDMAN; OLESEN, 1976).

Excluding the studies with galanthamine, most of the reports concerning AmAl biosynthesis were carried out in the 1960s or 1970s. Studies with *R. bifida* plants (FEINSTEIN; WILDMAN, 1976; LAURAIN-MATTAR; PTAK, 2016) indicate that 11-hydroxyvittatine (haemanthamine ring system) is formed by the addition of a hydroxyl to the vittatine. This molecule might be the montanine (**15**) and haemanthamine precursors, with biosynthesis of haemanthamine being more efficient (only a methylation of the hydroxyl function in C_3 of 11-hydroxyvittatine). For montanine formation the rearrangement of the vittatine ring system is necessary to promote the methylation of the oxygen in C_2 (BASTIDA *et al.*, 2011; OLESEN, 1976; WILDMAN; OLESEN, 1976).

Methyltransferases are essential for directing intermediates to specific biosynthetic pathways (CON, 2016). The preliminary characterization of the N4OMT

enzyme, a class I *O*-methyltranferase, was performed in the 60's, using crude enzymatic extracts of *Nerine bowdenii*. This study (MANN, JAY; FALES; MUDD, 1963) demonstrated the methylation but the enzyme was only recently identified in *Narcissus* sp. *aff. pseudonarcissus* by heterologous expression in *E. coli* (KILGORE *et al*., 2014).

Cytochrome P450 enzymes belong to a large enzyme family and catalyze a wide range of monooxygenation/ hydroxylation reactions in plant secondary metabolism. Their activities include C-C and C-O phenol couplings, C-C bond cleavages, methylenedioxy-bridge formations, demethylations and oxidative rearrangements of carbon skeletons (MIZUTANI; SATO, 2011). The C-C phenol coupling are key reactions for the formation of a fairly large number of pharmacologically active plant compounds (GESELL *et al*., 2009).

The P450 CYP96 family seems to be a young plant CYP family and the members can act in fatty acid hydroxylation and are known for the catalysis of ω, ω-1, and ω-2 positions (MIZUTANI; OHTA, 2010; NELSON, 2006). In *Arabidopsis* they are related to wax production which protect plants against water loss (GREER *et al*., 2007). In *Catharanthus roseus* cell cultures they are associated to the biosynthesis of terpenoid indole alkaloids (OUDIN *et al*., 1999). In *Narcissus*, the CYP96T1 gene is responsible for the *para-para'* phenol coupling reaction generating the haemanthamine and crinine carbon skeletons (KILGORE, 2015).

Although 4'-*O*-methylnorbelladine is the universal substrate in the biosynthesis of the AmAl, the *4OMT* and *CYP96T1* genes sequences were only identified in plants that produce galanthamine. In this study we have set up the conditions for *in vitro* plant regeneration and initiated the *N4OMT* and *CYP96T1* genes characterization in *R. bifida*. We have partially characterized the *N4OMT* and *CYP96T1* genes as well as their expression in different organs and culture types. In addition, we have correlated their expression to alkaloids production in different *R. bifida* wild type seedlings and adult plant as well as in regenerated plants, in different organs and culture conditions.

Experimental

Plant material and in vitro culture

Rhodophiala bifida plants were collected in Pelotas (RS-Brazil) (Latitude: -31.7719; Longitude: -52.3425; err: ± 51462 WGS84) in the year of 2015 (July). The bulbs were separated, washed in running water, pre-disinfected in 2% sodium hypochlorite for 3 hours, washed in sterile water and oven drying at 23ºC for 24 h. After this period, the bulbs were stored at room temperature and before transfer to *in vitro* culture or greenhouse, they were kept at 10°C and low humidity. The seeds of *Rhodophiala bifida* var. Spathacea were purchased commercially (Jelitto®, Germany).

Some of the bulbs were cultivated in the greenhouse, with a low organic mix (~1:3 organic: inorganic) of soil: sand: perlite (25: 40: 35, %), without incident light. They were irrigated every two days with water and nutritive solution each 10 days.

For the seeds, the disinfection started with alcohol 70% (1 minute) under agitation, sodium hypochlorite 3% active chlorine plus two drops of tween 20 per 100 mL, for 20 minutes under agitation, and washed with sterile water (3 times) inside of the laminar flow hood. They were then inoculated in water/agar medium, containing agar 7 g L^{-1}. The germination process took place at 20 °C (day) and 16°C (night), in the dark during the first 10 days. After this period seedlings were transferred to individual test glass tubes containing ½ MS medium, sucrose 15 g L^{-1}, PPM® 200 μL L^{-1} and agar 7 g L^{-1}. The medium was changed every three weeks, until the time of harvest for the analysis.

For *in vitro* culture, bulbs had their darkened external layers removed and were disinfected as follow: (i) agitation in alcohol 70% for 1 minute, (ii) agitation in sodium hypochlorite 8% plus tween 20 (2 drops per 100 mL) for 30 minutes and (iii) washed with sterile water (3 times). In laminar flow hood, the bulbs were dried on sterile filter paper, cut into tween scales (1.0 x 1.5 cm fragments) and placed in Petri dishes containing 20 mL of MS medium modified (KYTE *et al*., 2013) including MS macronutrients, inositol 100 mg L^{-1}, nicotinic acid 0.5 mg L^{-1}, thiamine 0.1 mg L^{-1}, pyridoxine 0.5 mg L^{-1}, casein 1 g L^{-1}, glycine 2 mg L^{-1}, phytagel® 2 g L^{-1}, PPM® 200 μL L^{-1}, sucrose 30 g L^{-1}, and pH regulated in 5.5 before autoclaving. Furthermore, growth regulators were sterilized by membrane filtration (Millipore® 22 μm) and added to the medium after autoclaving, naphthalene acetic acid (NAA) 0.2 mg L^{-1} and 6-

benzylaminopurine (BA) 1.5 mg L^{-1} according to a protocol previously established (REIS; PEDRAZZA; ZUANAZZI, 2014). The media were autoclaved at 121 °C, at 1 atm for 20 min.

After shoot regeneration, explants were transferred to flasks containing culture medium for multiplication. This medium consisted of MS medium salts with the addition of inositol 100 mg L^{-1}, nicotinic acid 0.5 mg L^{-1}, thiamine 0.5 mg L^{-1}, pyridoxine 0.5 mg L^{-1}, glycine 1 mg L^{-1}, agar 7 g L^{-1}, activated charcoal 5 g L^{-1}, PPM® 200 μL L^{-1} and sucrose 30 g L^{-1}, and pH 5.5 (KYTE *et al.*, 2013).

Once the multiplication process has started, plants were moved to MS liquid medium containing 100 mg L^{-1}, nicotinic acid 0.5 mg L^{-1}, thiamine 0.5 mg L^{-1}, pyridoxine 0.5 mg L^{-1}, glycine 1 mg L^{-1}, sucrose 30 g $^{L-1}$ and PPM® 200 μL L^{-1}, pH 5.5. The media containing the microplants were renewed every three weeks. After 90 days, the *in vitro* plants were acclimated to the greenhouse using the conditions described for the plants cultivated in this place.

The harvest occurred when greenhouse plants (wild and acclimated) were eight and six months old respectively. *In vitro* plantlets were harvested after 3 months of growth. For alkaloids extraction, plant material was oven dried at ± 40 °C and ground before chemical analysis. The samples used for the Quantitative Real Time-PCR were the same, but they were placed in liquid nitrogen immediately after harvest. For these analyses (chemical and molecular) plants were separated into roots, bulbs and leaves to study the organ-dependent gene expression or alkaloids content.

Nucleic acid extraction

Total RNA extractions were performed according to the protocol already recommended and used in Dr. P. Ratet Laboratory (Bâtiment 630, Plateau du Moulon, rue de Noetzlin, 91192 - Gif-sur-Yvette – France). In this way, wild plant bulbs during flowering season (100 mg) were selected and immediately frozen in liquid nitrogen for micronization until becoming powder. In sequence, was proceed the addition of TRIzol® reagent (Ambion), chloroform, isopropanol and 75% etanol were added at 4 °C, the supernatant removed, the pellet dried and dissolved in water (RNAse free), the mix was heated at 55 °C for 10 minutes to solubilize the RNA in the water. Afterwards,

the samples were treated with TURBO DNA-free™ Kit (Ambion) according with the manufacturer's recommendations.

Isolation of full-length cDNA and Cloning of the cDNA from Rhodophiala bifida

The full-length cDNA was generated by RT-PCR using SuperScript™ II Reverse Transcriptase Kit (Invitrogen) in presence of Ribolock RNase Inhibitor (Thermoscientific) and beginning with 2 µg of total RNA.

All the primers were designed based on sequences conserved at the amino acids and nucleotides level. Sequence comparisons were done with Blast search (http://www.ncbi.nlm.nih.gov/BLAST/) using sequences from the Amaryllidaceae family, OMT and CYP96T as criteria. For the *N4OMT* the forward primer was ATGGGTGCTAGCATAGATGATTAT and the reverse primer TCAATAAAGACGTCGGCAAATAGT. For the CYP96T the forward primer was CCATGGCCACTTCTTCTTCAGCATG and the reversed primer CCTCACATGACTGATCTCTTTCTAA. PCRs were done using Phusion High-Fidelity DNA polymerase (Thermo Scientific, USA) in a protocol containing 1 pre-incubation cycle (95°C, 5 min) and 35 amplification cycles [(denaturation: 95 °C, 30 s), (hybridization: 55 °C, 30 s), (elongation: 72 °C, 1 min)].

The vector used for cloning the PCR products was Plasmid pGEM®-T Vector (Promega – USA) and the One Shot® TOP10 Chemically Competent *E. coli* was used for plasmid amplification.

Quantitative Real Time-PCR (qRT-PCR) for gene expression analysis in different organs and plant cultivations

To isolate the RNA, we proceed with the technique described before. The qRT-PCR reactions were performed using the LightCycler FastStart DNA Master SYBR Green I kit on a Light Cycler 480 II device agreeing to producer's manual (Roche). Cycling conditions were as follows: 1 pre-incubation cycle (95°C, 10 min), 40 amplification cycles [(denaturation: 95 °C, 10 s), (hybridization: 60 °C, 15 s), (elongation: 72 °C, 15 s)], 1 melting curve cycle [(denaturation: 95 °C, 15 s), (hybridization: 55 °C, 1 min), (denaturation: 95 °C)], 1 cooling cycle (40 °C, 30 s).

Cycle threshold and primer specificities were performed with the LightCycler 480 software release 1.5.0 SP4.

Primer efficiencies were calculated with LinReg PCR: Analysis of Real-Time PCR Data, version 11.1. For gene expression normalization actin gene primers were used as reference gene. These primers specifically designed to monitor *R. bifida ACTIN* genes expression were TCCATCATGAAGTGTGATGTTGATAT as forward primer and CCTCCAATCCAGACACTGTACTT as reverse primer.

To measure the expression of the *CYP96T* genes, the primers used were CTGCTTGGAATTCAAGCCCGA as forward primer and ATGCCCCTGGACCATCCGAA as reverse primer. For the N4OMT CGTCAGGGTTGGAGGAGCGAT (forward primer) and ATCGCTCCTCCAACCCTGACG (reverse primer) were used.

Alkaloids extraction and HPLC analysis

The extraction of total alkaloids was performed according to (GEORGIEVA *et al*., 2007) with small modifications. We used 100 mg of plant material and the extraction was initiated with 2% sulfuric acid (V/V) on an ultrasound bath for 4 hours. The samples were gravity filtered and the supernatant washed with ethyl ether (3 x 100 mL). The aqueous fraction was basified with 25% ammonium hydroxide (V/V) until pH 9. This basic aqueous solution was partitioned with ethyl acetate (3 x 100 mL). The organic residue was filtered over anhydrous sodium sulfate and the volume was reduced to the residue in a rotatory evaporator. The alkaloid rich residue was resuspended in methanol 2 mL, filtered through a membrane of 0.45 μm (Millipore ®) and analyzed.

Liquid chromatographic analyzes were performed on a Waters Alliance e2695 with a diode arrangement detector (PDAWaters 2998), and software Empower 3 HPLC (Waters®) for data acquisition and treatment. The chromatographic column employed was reverse phase (Synergi Polar Phenomenex, 80 Å, 4 μm, 250 x 4.60 mm) and coupled to a reversed-phase pre-column (Security Guard Cartridges™ Fusion; 4 x 3.0 mm). The elution system used was composed by mobile phase A with water: trifluoroacetic acid (TFA) (100: 0.01, V/V) and mobile phase B, acetonitrile: TFA (100: 0.08, V/V). The flow rate used was 0.5 mL min^{-1}, temperature for the sample compartment 20 °C and

the gradient start with solvent A at 85% (0-13 min), 82% (2 min); 79% (2 minutes), 77 % (2 min); 100% of solvent B (hold for 2 min), 85% of solvent A (for the next 2 minutes), totaling 22 minutes of analysis using the wavelength of 290 nm for montanine quantification.

Alkaloid identification by GC-MS

About 100 mg of each extract sample were dissolved in 100 µl of methanol and injected directly into the GC-MS apparatus (Agilent Tecnologies 6890N coupled with MSD5975 inert XL) operating in the EI mode at 70eV. A Sapiens-X5 MS column (30 m x 0.25 mm i.d., film thickness 0.25 µm) was used. The temperature gradient performed was the following: 2 min at 100 ºC, 100-180 ºC at 15 ºC.min^{-1}, 180-300 ºC at 5 ºC.min^{-1} and 10 min hold at 300 ºC. The injector and detector temperatures were 250 ºC and 280 ºC, respectively, and the flow-rate of carrier gas (He) was 1 ml. min^{-1}. A split ratio of 1:10 was applied and the injection volume was 1µl.

Statistical analysis

For the chemical analysis we used at least three biological replicates for all of the samples and two technical replicates of each biological replicate. For the qRT-PCR, at least three biological replicates were used for all of the samples and two technical replicates of each biological replicate. The data were analyzed using analysis of variance (ANOVA) and means were compared using the Tukey test, with a 5% level of significance (P <0.05).

Results and discussion

Characterization of the RbCYP96T and RbN4OMT genes

The AmAl represent a very large group of molecules present exclusively in this plant family. More than 300 structures were already described, encompassing a range of biological activities. Although the initial part of the biosynthetic pathway has been described, most of the enzymes and the corresponding genes remain unknown (KILGORE *et al.*, 2016a). For example, molecules such as montanine have well described biological activities, but molecular data date back from decades ago and

important genes of this pathway have still to be described in order to boost pharmaceutical studies.

The cDNA fragments were amplified using RNA extracted from *Rhodophiala bifida* bulbs RNA. Primers were designed based on conserved regions of CYP96T1 and N4OMT *Amaryllidaceae* nucleotides and amino acids publicly available sequences (NCBI; GenBank; (GEER *et al*., 2009).

Previous studies demonstrated that the methyltransferase enzyme N4OMT is responsible for the methylation in the alkaloid norbelladine resulting in the formation of AmAl 4'-*O*-methylnorbelladine (KILGORE *et al*., 2014). In order to identify the corresponding gene in *R. bifida*, we designed oligonucleotides in the regions conserved between *Amaryllidaceae* genes representing close homologs of the five *Narcissus N4OMT* genes (*NpN4OMT1* to *NpN4OMT4*; Sup Fig. 1). For this gene the oligonucleotides included the AUG and stop codons. The amplified *R. bifida* fragment was cloned and sequencing of the 744 bp fragment from several clones provided a unique full length cDNA sequence indicating that only one gene is expressed in the organ analyzed. The putative *R. bifida N4OMT* gene shows respectively 93, 92, 92, 92 and 93% identity with the *Np*N4OMT1 (KJ584561.1), *Np*N4OMT2 (KJ584562.1), *Np*N4OMT3 (KJ584563.1), *Np*N4OMT4 (KJ584564.1) and *Np*N4OMT5 (KJ584565.1) genes corresponding to the *N*. aff. *pseudoNarcissus* N4OMT protein (KILGORE *et al*., 2014); Supplementary Material – Fig. Supl. Mat. 1). The 239 amino acid *R. bifida* protein shows 92 to 93% identity to the *pseudonarcissus* protein. Thus, the gene *R. bifida* cloned here was considered as the Norbelladine 4'-*O*-Methyltransferase putative ortholog and was named *RbN4OMT*. The nucleotide and protein sequences were deposited in the GenBank database (with accession number xxxx and xxx).

The CYP96T1 enzyme catalyzes the transformation of 4'-*O*-methylnorbelladine to noroxomaritidine, leading to the formation of noroxomaritidine derivatives, like haemanthamine (KILGORE *et al*., 2016b) and montanine. In order to characterize a *R. bifida CYP96T1* gene, we also tried to designed oligonucleotides in conserved regions of the *pseudonarcissus CYP96T1* genes. The coding sequence of this gene is 1539 bp long (513 aa). However only *N. pseudonarcissus* highly homologous sequences

corresponding to CYP96T3 (AMO65743.1), CYP96T2 (AMO65742.1) and Noroxomaritidine synthase Cytochrome P450 96T1 (A0A140IL90.1) are present in Genebank and do not allow defining conserved regions. Using genomic data from *Lycoris*, we found a partial gene sequence homologous to the *PseudoNarcissus* gene and covering 415bp of the 3' part of the coding sequence. Using a combination of oligonucleotides designed against *CYP96T1* genes including the *Lycoris* sequence, we amplified a *Rhodophiala* cDNA sequence covering the 500 bp (166 aa) of the putative *R. bifida CYP96T1* sequence. This sequence covering 30% of the 3' part of the *PseudoNarcissus CYP96T1* coding sequence shows 83% nucleotide identity and 81% identity (90% similarity) at the aa level to the p*seudoNarcissus* CYP96T (KILGORE *et al*., 2016b) (Supplementary Material – Fig. Supl. Mat. 2). The partial sequence corresponding to a putative *para-para'* C-C phenol coupling cytochrome P450 was named *Rb*CYP96T and the sequence was deposited in the GenBank database (accession number xxxx).

Expression of RbCYP96T and RbN4OMT in R. bifida different plant cultivations and organs

The expression of *RbCYP96T* and *RbN4OMT* genes were studied in different organs and types of cultures by semi-quantitative RT-PCR. The RNA samples used to perform the RT-PCR were isolated from roots, bulbs and leaves of *R. bifida* cultivated in greenhouse conditions for wild type or acclimated *in vitro* regenerated plants, or for plantlets grown *in vitro* from germinated seeds.

The RbN4OMT gene was expressed in almost all the tissues and different plant culture conditions Fig. 3*(a)*. The expression level was the highest for bulbs of germinated and acclimated plants but not for the WT plants. The level of expression in the other organs and modes of cultivation was lower with almost no expression detected in leaves of acclimated plants.

These results suggest that the RbN4OMT gene was expressed in developing plants in which there is need for constant growth. This indicates that during germination and acclimatization, the highest level of expression could result in methylation of Norbelladine (**9**) to form 4'-*O*-methylnorbeladine (**10**) in bulbs.

Studies initiated in the 80's showed that alkaloids may influence plant growth as growth stimulators and regulators. However, the most important biological function is the protection of plant cells, from physical stresses (UV-light and heat), against pathogens and herbivores and can help in adapting the plant to the local environment (ANISZEWSKI, 2015). Thus, the expression of *RbN4OMT* may be related to this adaptation to the local environment and growth for the germinated and acclimated plants analyzed.

We believe that in the WT bulbs the activity remained basal due to the fact that there is no cell zone that continues the cell divisions indefinitely and there is no additional vascular tissue formation when stem width growth is complete (REES, 1972), slowing the development of the bulb. Interestingly in the WT plant the highest expression is detected in the root and correlate with the pattern of expression seen for the *RbCYP96T* gene.

In order to obtain a better visualization of the RbN4OMT gene expression in *R. bifida* plants, the analysis was done using the different organs of the WT (Fig. 4). This analysis indicates that the RbN4OMT gene is expressed in roots, bulbs, leaves, old leaves and flowers but that they were no statistical differences in the level of expression for these tissues. This may correlate to the low level of expression detected in these WT plants, may be in relation with their age. This is different from the results found in *N.* sp. aff. *pseudonarcissus* WT, collected during the flowering season. In this plant the highest expression was detected in bulbs, followed by inflorescences (intermediate results) and leaves (close to zero) (KILGORE *et al*., 2014).

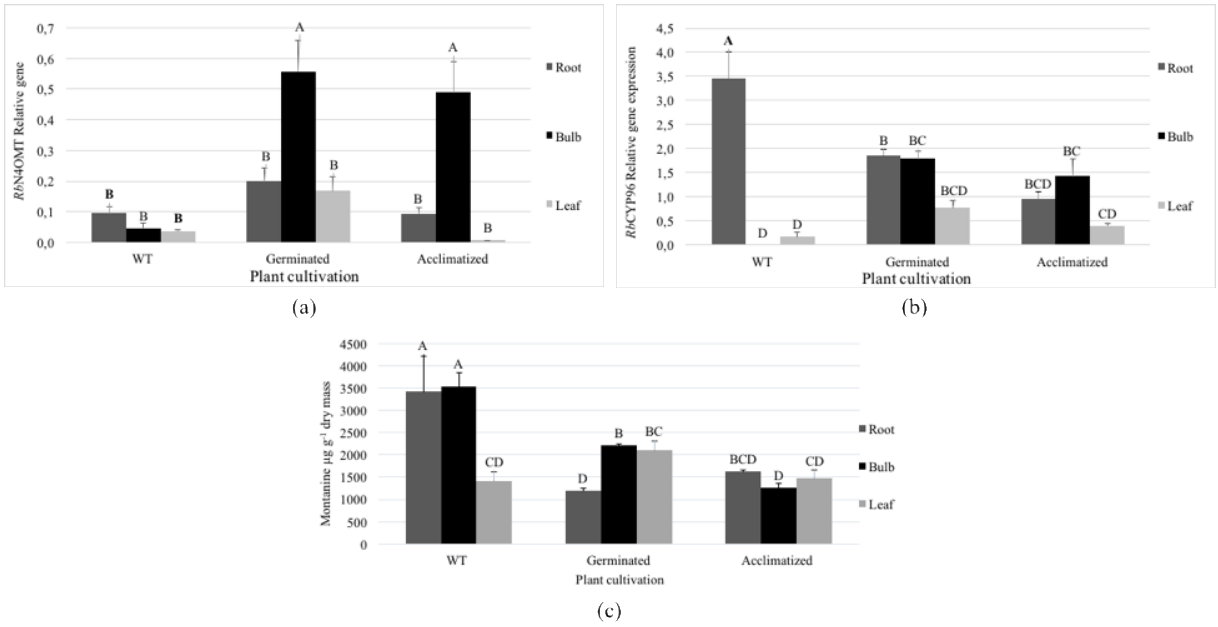

Figure 3. Gene expression and montanine accumulation. (a) *RbN4OMT* and (b) *RbCYP96T* relative expression when using the actin gene as reference gene, in different plant organs and culture conditions. (c) Montanine content in distinctive organs and different plant growth conditions of *R. bifida* plants. The results shown are the means of the replicates; bars represent SE. Distinctive letters indicate significant differences according to the Tukey test ($P < 0.05$).

In *R. bifida* plants, the *RbCYP96T* gene was expressed in the majority of the tissues and cultures analyzed (Fig. 3b). Expression was the highest in the roots of the WT plants with nearly undetectable expression in bulbs. In acclimated plants the highest level of expression was detected in bulbs followed by roots and leaves. In germinated plants, the highest expression was detected in roots and bulbs followed by leaves. Statistical analysis of these results did not allow differentiating these organs for AmAl production.

When studying the *RbCYP96T* gene expression in the different wild type plant organs (Fig. 4 *b*), the highest expression was observed in roots and was statistically different from the other organs.

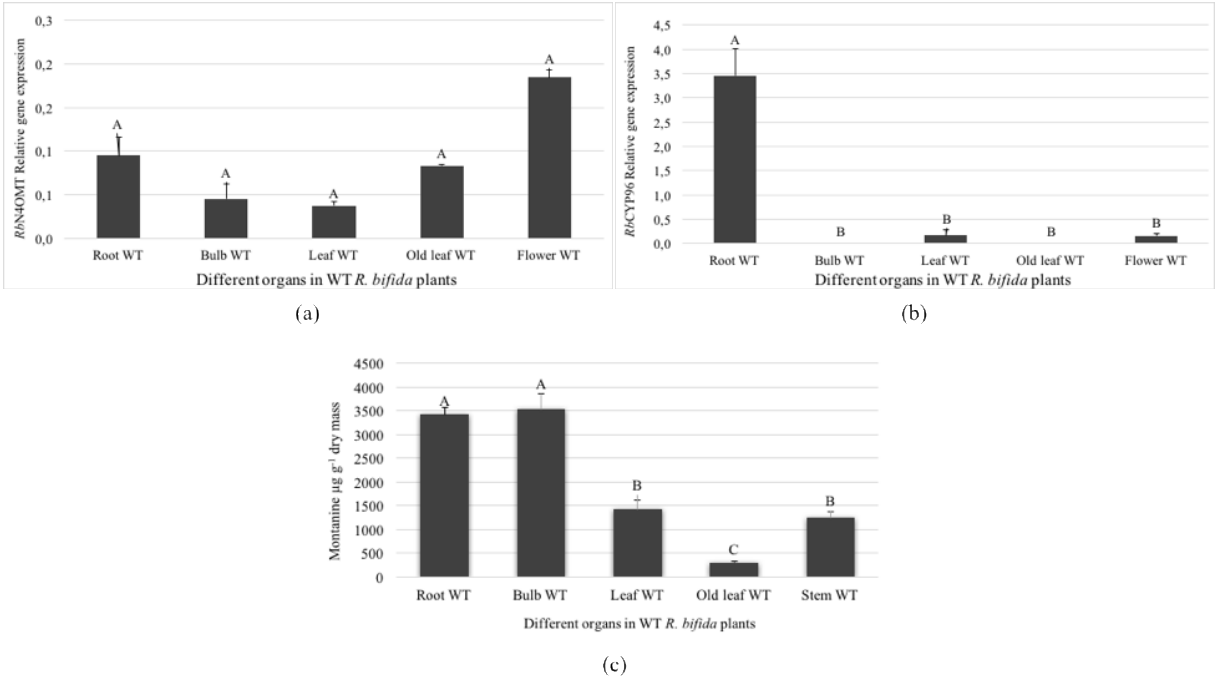

Fig. 4 Gene expression and montanine accumulation in *R. bifida* plants. (a) *RbN4OMT* and (b) *RbCYP96T* relative expression in different organs of WT plants, using the actin gene as reference. (c) Montanine content in distinctive organs of WT plants. The results shown are the means of three replicates; bars represent SE. Distinctive letters indicate significant differences according to the Tukey test ($P < 0.05$).

Alkaloids extraction, identification and quantification

Next, we wanted to correlate the gene expression to the presence of the AmAl production. First, we evaluated the linearity of the quantification method by constructing three linear curves on three different days with the solution containing montanine in seven different concentrations in the range of 120 to 2200 μg mL^{-1}. The linearity curves showed good linear correlation (with $R^2 = 0{,}999$, and linear equation of $y = 13036x - 371135$) and could be used to quantify AmAl in our extracts.

The measurement of the montanine content using HPLC analysis (Fig. 3c and Table 1) showed that the highest production was found in bulbs and roots of wild plants (3527.60 and 3424.75 μg g^{-1}, respectively), followed by bulbs of the seedlings (2212.75 μg g^{-1}). The germinated leaves (2097.50 μg g^{-1}), acclimated roots and leaves (1626 and 1473.44 μg $^{g-1}$, correspondingly) plus WT leaves (1419.19 μg g^{-1}). The lowest levels of montanine were detected in the roots of the germinated plants (1193,33 μg g^{-1}) and in bulbs of the acclimatized plants (1259,11 μg g^{-1}).

Table 1. Montanine content (μg g^{-1}) found in the organs of *Rhodophiala bifida* plants culture in different growth conditions.

Organ	Growth conditions		
	Germinated	Acclimatized	WT
Root	1193.33 D	1626.00 BCD	3424.75 A
Bulb	2212.75 B	1259.11 D	3527.60 A
Leaf	2097.50 BC	1473.44 CD	1419.19 CD

* Means not followed by the same letter differ from each other by the Tukey test, at 5% error probability. Germinated: *in vitro* grown seedling; Acclimated: greenhouse acclimated regenerated plants; WT: greenhouse grown plants.

Interestingly the measurements done separately for the wild plants tissues (Fig. 4c and Table 2) indicated that the amount of montanine (3527.60 and 3424.75 μg g^{-1}, respectively) was the highest in the bulbs and the roots, being about 2.5 x more important than in the leaves and the stems (1419.13 and 1252.38 μg g^{-1}). The lower yield was found in old leaves (302.77 μg g^{-1}) almost 12 x smaller than the amount produced by bulbs.

Table 2. Montanine analysis of content found in the different organs of wild *Rhodophiala bifida* plants.

Organs	Montanine (μg g^{-1})	SD
WT- Roots	3424.75 A	± 802.27
WT- Bulbs	3527.60 A	± 333.95
WT- Leaves	1419.13 B	± 203.42
WT- Old leaves	302.77 C	± 19.94
WT- Stem	1252.38 B	± 127.70

± standard deviation (SD).*Means not followed by the same letter differ from each other by the Tukey test, at 5% error probability.

In a study carried out with *N. pseudonarcissus* L. cv. Carlton wild plants (LUBBE *et al*., 2013) production of alkaloids in bulbs, roots and leaves during the growth season, was the highest before the flowering and the bulbs had the lowest concentrations of the compounds galanthamine, haemanthamine and narciclasine.

In *Licoris aurea* plants alkaloids content increased during the growing season. The galanthamine content was the lowest in leaves and the highest in bulbs. For this plant around 99.5% of galanthamine was detected in bulbs and roots, with the highest level identified in roots and bulbs at the time of leaves withering in autumn (RU *et al*., 2013). This is similar to our results in which the highest montanine content was found

in the WT roots and bulbs, agreeing also partially with the results obtained for *Crinum macowanii* plants, were bulbs had the highest alkaloid content, companied roots, flower stalks and leaves (ELGORASHI; DREWES; VAN STADEN, 2002).

We showed above that the expression of the *Rb*N4OMT gene (Fig. 3) was rather low in WT plants with the expression being the highest in the roots. In these plants the level of montanine was equivalent and rather hight in the root and the bulbs but lower in the leaves. We can hypothesize that the production of alkaloids takes place earlier during plant development, in agreement with the expression results of the germinated or acclimated plants. After accumulation in the root and bulbs of older plants the gene expression became lower in these WT organs. This is in agreement with the results obtained with *Lycoris sprengeri* bulbs, where the starch gene expression levels decreased as the bulb diameter increased (CHANG *et al*., 2013).

The *RbCYP96T* gene (Fig. 3) is expressed at high level in the WT root but was below detection level in the bulb, despite the high content of montanine in these tissues. Since the bulb is an underground storage organ, it is again possible that biosynthesis of the alkaloids occurs earlier during bulb development in order to protect the plant's carbohydrate resources from herbivores and microorganisms (RUIZ; WARD; SALTZ, 2002; TAKOS; ROOK, 2013). It cannot also be excluded that a second CYP96T gene is expressed specifically in the bulb in this plant, and was not detected in our experiments.

The germinated and acclimated samples (Fig. 3) presented intermediate expression levels for the *RbCYP96T* gene as well as intermediate alkaloid accumulation. As indicated above, since these plants were developing plants (growing seedlings and regenerated *in vitro* plants developing in the greenhouse), the biosynthetic genes expressed moderately. These were small plants with short and thin roots (germinated), little but growing bulbs and thin expanding leaves. It should be also noticed that the increase of the molecules seen in an organ without or with low level of gene expression can result from the transport from other organs as was observed in *Narcissus* Carlton (LUBBE *et al*., 2013).

The alkaloid biosynthesis and content of *Leucojum aestivum* depends on the geographical latitude of the studied populations and on the soil fertility level (BOGDANOVA *et al*., 2009; GORINOVA *et al*., 1993). Our results suggest in addition that the developmental stage of the plant can modify the profile of the alkaloids, as observed in our analysis for the *R. bifida* plants.

The amount of the different compounds detected by GC–MS were expressed as a percentage of the total alkaloids present in *R. bifida* wild plants (TIC for Total Ion Current), grown in a greenhouse. The structural types of the alkaloids were identified by comparing their GC-MS spectra and Kovats Retention Index (RI) values with those of authentic Amaryllidaceae alkaloids previously isolated and identified by spectrometric methods (NMR, UV, CD, MS) in previous analysis. The MS spectra were deconvoluted by AMDIS 2.64 software (NIST). The GC–MS peaks area depends on the concentration and intensity of the equivalent compound of its mass spectral fragmentation.

Montanine, possess a 5,11-methanomorphanthridine nucleus and differ from the other montanine type alkaloids because of the substituents at C-2 and C-3 (BASTIDA; LAVILLA; VILADOMAT, 2006). According to our study, montanine (**15**) was present in all organs, in a range from 49.7 to 88 % of TIC. This compound is thus the most abundant alkaloid of the plant. The highest amount as deduced from the major peak was found in roots (74-88) and the lowest amount in leaves (53.7-56.3). Results published before with *R. bifida* bulbs, indicated that the percentage of montanine reached 92% of the alkaloids extracts (ANDRADE *et al*., 2015). Our results differed from this study in which bulbs had only trace amounts (less than 0.20 %) of vittatine, deoxytazettine, tazettine, pretazettine and 3-epimacronine.

The amount of tazettine (**21**) in our study was in a range of 1.3 to 13.1% of TIC, with the lowest amount in bulbs (1.3 to 2.9%) and the largest amount in leaves (13.1%). This compound is a part of the tazettine-type alkaloids, derived from the 2-benzopyrano[3,4-c]indole skeletons, widely reported in *Narcissus* and known as an extraction artifact of pretazettine (BASTIDA; LAVILLA; VILADOMAT, 2006).

The alkaloid vittatine or crinine (**13**) is an intermediate in narciclasine synthesis and is thus involved in the montanine formation. It was found in small concentrations in bulbs and leaves, (BASTIDA; LAVILLA; VILADOMAT, 2006).

Pancracine is the other molecule identified in all the tissues analyzed. It belongs to the montanine-type alkaloids identified in the 60's in *Pancratium maritimum*, *Narcissus poeticus* and *R. bifida* (WILDMAN; BROWN, 1968). The amount of this compound is ranging from <0.1 to 13% of TIC, with the lowest yield in roots (<0.1-4.3%) and bulbs (1.3-3.3%) and leaves with about 11.8 to 13% of TIC. This and the other related molecules indicated in Table 3, were not found in the study carried out in 2015 with *R. bifida* bulbs (ANDRADE *et al*., 2015). We believe that these differences may result from different harvesting times, places, soil, variety or from different chemotypes related to the species sampled.

8-*O*-demethylmaritidine was detected in the genus *Galanthus* (KAYA *et al*., 2011) and like trisphaeridine (**17**), 5,6-dihydrobicolorine (**18**), and other unidentified molecules were found only as traces in our study. The molecule 5,6-dihydrobicolorine (**18**) was identified for the first time in *Narcissus bicolor* and belongs to the phenanthridine type alkaloids (JIN; XU, 2013; VILADOMAT *et al*., 1990).

Ismine (**16**) and trisphaeridine (**17**) are considered as catabolic products from the haemanthamine- type skeleton (BASTIDA; LAVILLA; VILADOMAT, 2006). Galanthindole (**20**) contains a non-fused indole ring and might represent an artifact of homolycorine or tazettine-type derivatives (UNVER *et al*., 2003).

O-methyltazettine (**20**), 3-*O*-acetylpancracine (**23**) which possess a montanine type nucleus derived from pancracine (WILDMAN; BROWN, 1968) and 3-epimacronine (**24**) identified in *Sperkelia formossisima* in the 60's (WILDMAN *et al*., 1967) were detected as traces in our study.

Other molecules were found in low amount and some of them could not be identified in our study (Table 3).

Table 3. Alkaloids found in wild *R. bifida* plants and are expressed as percentage of TIC in each organ using GC-MS analysis.

Compound	Rt (min)	RI	$[M]^+$	Bulbs	Leaves	Roots
Ismine (**16****)	20.61	2267.7	257	<0.1 -1.0	<0.1 -1.0	<0.1
Trisphaeridine (**17**)	20.92	2286.6	223	<0.1	<0.1	<0.1
5,6-Dihydrobicolorine (**18**)	21.62	2330.1	239	<0.1	<0.1	-
m/z 270[*a]; [M = 343][*b]	23.57	2454.5	270	-	-	<0.1
m/z 238[*a]; [M = 271][*b]	23.59	2455.2	238	0.6-1.0	1.2	<0.1
m/z 288[*a]; [M = 289][*b]	23.70	2463.0	288	-	<0.1	-
Vittatine (crinine) (**13**)	23.89	2475.9	271	1.0-1.22	<0.1	-
Galanthindole (**19**)	24.23	2498.3	281	<0.1-2.1	<0.1-0.8	-
8-*O*-Demethylmaritidine (**12**)	24.40	2509.6	273	<0.1	-	-
O-Methyltazettine (**20**)	25.81	2604.7	345	<0.1-3.3	<0.1	-
Montanine (**15**)	26.36	2641.4	301	49.7-77.6	53.7-56.3	74-88.0
Tazettine (**21**)	26.52	2652.4	331	1.3-2.9	13.1	8.5-9.3
m/z 252[*a]; [M = 343][*b]	26.61	2658.2	343	-	11.8	-
m/z 331[*a]; [M = 331][*b]	26.70	2664.4	331	<0.1	-	-
Pancracine (**22**)	27.21	2698.0	287	1.3 -3.3	11.8-13.0	<0.1-4.3
3-*O*-Acetylpancracine (**23**)	27.77	2736.1	329	-	1.6	-
m/z 270[*a]; [M = 329][*b]	28.42	2779.3	270	-	<0.1	-
3-Epimacronine (**24**)	28.84	2807.6	329	<0.1	<0.1 -1.1	<0.1
m/z 252[*a]; [M = 369][*b]	29.44	2848.0	252	-	<0.1	-
m/z 252[*a]; [M = 369][*b]	29.67	2863.1	252	-	4.1-5.0	-
m/z 252[*a]; [M = 387][*b]	31.07	2957.3	252	1.1-2.6	7.2-10.3	<0.1

[*a]base peak; [*b]possible molecular ion peak. **The molecules represented by bold numbers in brackets are in the biosynthesis pathways (Fig. 1 and 2) or in the supplementary material (Fig. Supl. Mat. 3). Three samples were analyzed for each organ. Values are indicated as the proportion of each individual molecule in the alkaloid fractions.

Conclusion

We present here a complete protocol for regeneration using *R. bifida* twin scales, in which the plant can be produced for a long time in the laboratory. We also present the necessary management to transfer *in vitro* plants to soil and to grow them in a greenhouse. These conditions can be adopted for future large-scale productions.

We have identified a full-length *N4OMT* and a partial of *CYP96T1* P450 cDNAs, and designed functional primers for the *Amaryllidaceae* actin gene that can be used in expression studies. This finding may help studying the regulation of *Amaryllidaceae* alkaloids production. This is also the first step in the molecular characterization of the *R. bifida* montanine biosynthetic genes and we believe it will boost the studies related to the production of this compound.

Our gene expression study in the different tissues and culture conditions represents a founding study for future research in order to know how gene expression and alkaloid production can be correlated and enhanced by various treatments such as elicitor treatments. Our study suggests that *RbN4OMT* best levels of expression were in bulbs of germinated and acclimated plants and that *RbCYP96T* highest expression was found in wild plants roots followed by germinated plants.

The best montanine production (88% of TIC in roots when identified by GC-MS) was observed in bulbs and roots of wild plants.

Supplementary material

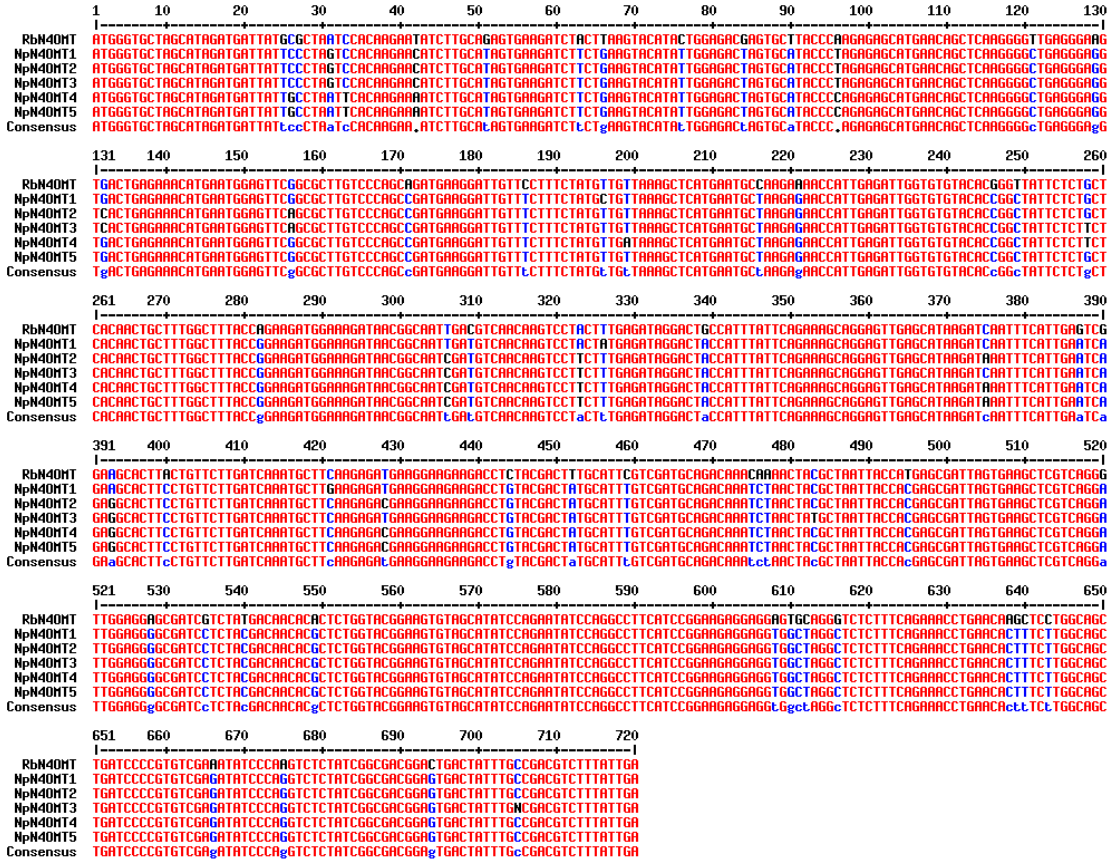

Fig. Supl. Mat. 1. Multi-alignment analysis (CORPET, 1988) showing high identity (in red) between the nucleotide sequence found in our study, *Rb*N4OMT, to N4OMT reported in *Narcissus* aff. *pseudoNarcissus* MK-2014 sequences (KILGORE *et al*., 2014) sharing a similarity of 93, 92, 92, 92 and 93% with *Np*N4OMT1, *Np*N4OMT2, *Np*N4OMT3, *Np*N4OMT4 and *Np*N4OMT5.

Fig. Supl. Mat. 2. Multi-alignment analysis showing high identity (in red) between the protein sequence found in *R. bifida*, *Rb*CYP96, when correlated with *N.* aff. p*seudoNarcissus* CYP96 protein sequences (KILGORE *et al.*, 2016b). The identity was 84% identity with CYP96T3, CYP96T2 and Noroxomaritidine synthase Cytochrome P450 96T1.

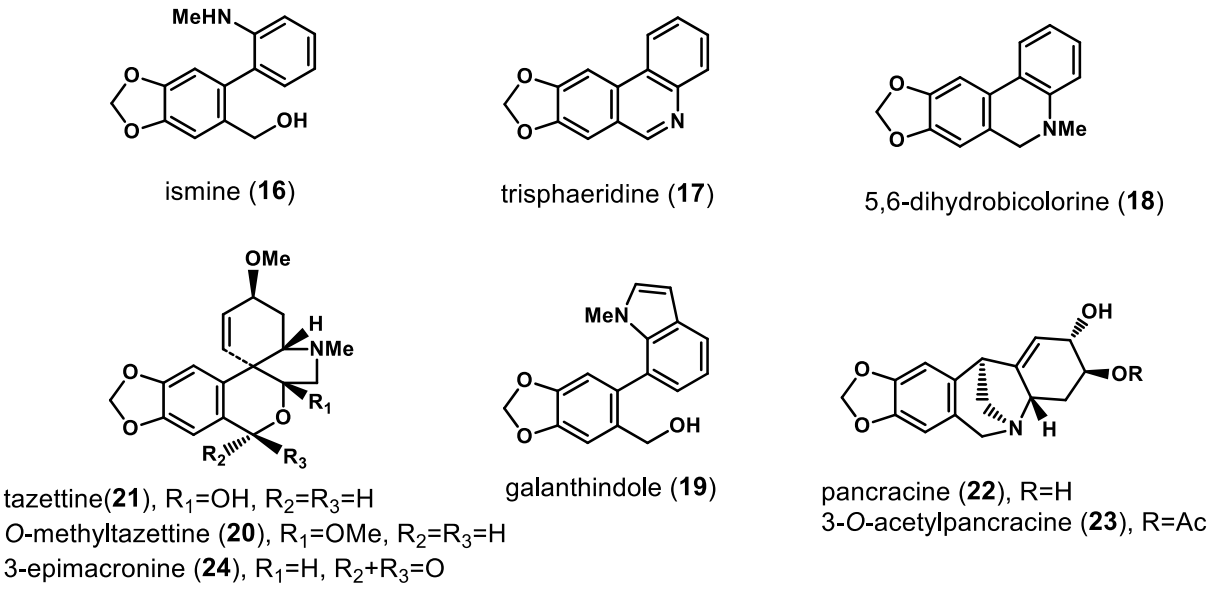

Fig. Supl. Mat. 3. Identified molecules found in wild plants of *R. bifida* analyzed by GC-MS.

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CAPÍTULO IV

Trifolium pratense L.: friable calli, cell culture protocol and isoflavones content in wild plants, *in vitro* and cell cultures analysed by UPLC

Introduction

Trifolium pratense L. (red clover) is a perennial plant belonging to the Fabaceae family, rich in polyphenolic compounds such as daidzein, genistein, formononetin and biochanin A (Fig. 1-*a, b, c, d*). These isoflavones have proven use in relieving menopausal symptoms, preventing osteoporosis, benign prostatic hypertrophy, hormone replacement therapy, cardiovascular disease, hypertension, and hormone-dependent tumors (Heinonen *et al*. 2002; Beck *et al*. 2005; Wuttke *et al*. 2006; Nissan *et al*. 2007; Ceolato *et al*. 2012; Ercetin *et al*. 2012; Çölgeçen *et al*. 2014; Spagnuolo *et al*. 2014; Xu *et al*. 2015).

The isoflavonoids are a distinct class among flavonoids originated from the phenylpropanoid pathway (Figure 1) and mosto f these molecules are formed by dividing the route provided into two branches, according to their key intermediates, genistein is formed by naringenin and subsequently, by means of the enzyme isoflavone 4'-*O*-methyltransferase can form the biochanin A. In the case of daidzein, the intermediate is the liquiritigenin and with the action of the same isoflavone 4'-*O*-methyltransferase, originates formononetin (Du *et al*. 2010; Kanehisa *et al*. 2017). The phytoestrogenic activity related with the isoflavones is based on the structural similarity with estrogens 17β-estradiol (Fig. 1-*e*), being able to act as agonists or antagonists, being their action determined in a dose-dependent manner (Wu *et al*. 2003).

The use of wild clover plant extracts often fails to meet demands because of the very large variability in the isoflavones content. One of the major problems faced in the activity tests with these extracts would be the lack of a chemical standardization in the contents of these phytoestrogens (Spagnuolo *et al*. 2014).

Such fluctuations would occur due to seasonal variations, production at specific stages of development, stress, nutrient availability or soil conditions. Thus, in the last decades much has been studied regarding the possibility of production of plants *in vitro* as an alternative of commercial interest in obtaining secondary metabolites where all the physical and chemical parameters to which the plant can access can be controlled, vou reducing the interferences and being able to predict the molecular content of the

isoflavones for the use in the manufacture of the drugs with the highest standardization of the molecules (Verpoorte *et al*. 2002; Booth *et al*. 2006).

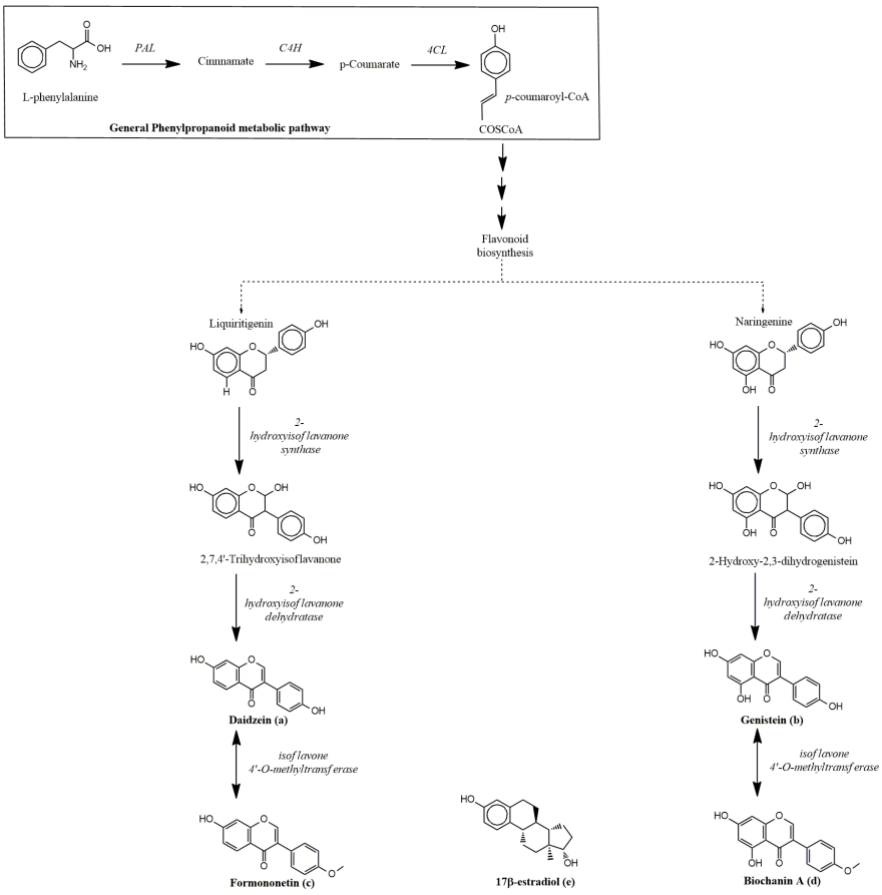

Figure 1. Summarized biosynthetic route of isoflavonoids showing the general phenylpropanoid metabolic pathway and the Isoflavonoid pathway with the best known isoflavone molecules: daidzein (**a**), genistein (**b**), formononetin (**c**), biochanin A (**d**) and the estrogen 17β-estradiol (**e**) for molecular comparison. Adapted from (Du *et al*. 2010; Kanehisa *et al*. 2017). Abbreviations: PAL phenylalanine ammonialyase, C4H cinnamate-4- hydroxylase, 4CL 4-coumarate CoA ligase.

Cell cultures are also another alternative for the production of metabolites of high commercial value on a large scale because, unlike field cultivation, in small space there is possibility of producing a large quantity of material, with different lines producing specific molecules, its possible to maintain for long periods, without great maintenance required, there is ease of extraction of the chemical constituents because they are in the format of cellular aggregates and it is a possible to elicitate the cultures for greater production of the target compounds (Satdive *et al*. 2015).

The protocols for *in vitro* micropropagation and cell culture are necessary for the application of biotechnological methods, aiming at transformation methodology and

plant breeding for secondary metabolites production in our future studies. Considering this scenario, the objective of this work was to establish a protocol for *in vitro* micropropagation of *Trifolium pratense* var. URS-BRS Mesclador plants as well as a protocol for suspended cell cultures, and to quantify isoflavone content of the wild, *in vitro* plants and the cell cultures.

Materials and methods

Plant material

The wild plants and seeds of *Trifolium pratense* var. URS-BRS Mesclador were donated by Dr. Miguel Dall'Agnol (Faculty of Agronomy - UFRGS). The voucher specimen is deposited in the Herbarium Alarich Rudolf Holger Schultz-HAS, in the Museum of Natural Sciences - Zoobotanic Foundation of Rio Grande do Sul, under registration HAS 87114, number 4291 (10/14/1986), and the point of collection was made in the latitute: -30.0331 and longitude: -51.23 [err: ± 29946 WGS84], by the Faculty of Agronomy (UFRGS), Brazil.

Disinfestation and germination

After receiving the seeds, the disinfestation protocol was started using ethanol 70% (1 minute) under stirring, sodium hypochlorite 11% with 2 drops of tween 20 for each 100 ml of solution (5 minutes, under stirring) and washed with distilled and autoclaved water (thrice), in laminar flow, for the removal of the disinfestation solutions. After this procedure, the seeds were dried on sterile filter paper for 30 minutes and then inoculated.

The seeds were inoculated in MS medium (Murashige and Skoog 1962) supplemented with inositol 100 mg L^{-1}, phytagel® 1.5 g L^{-1}, sucrose 30 g L^{-1}, thiamine, nicotinic acid and pyridoxine at 0.5 mg L^{-1}, glycine 1 mg L^{-1} and *Plant Preservative Mixture*® (PPM) at 0,2 g L^{-1}. The culture medium was used containing 100%, 50% and 25% of the salts of the MS médium (macro and micronutrientes). The pH was adjusted to 5.8 and autoclaved for 20 minutes at 121 °C and 1 atm.

Subsequently to inoculation, the seeds were brought to the dark environment where they remained for 15 days and then transferred to the light (photon flux of 40

μmol m^{-2} s^{-1}), 25 ± 2 º C, for another 15 days. After 7 and 30 days of light exposure, the cultures were evaluated for the percentage of contamination, to determine the efficiency of the disinfestation protocol, and the percentage of germination in the different concentrations of medium used.

Multiplication, induction of friable callus and initiation of cell culture

Passed 30 days of seed inoculation, seedlings emerged were transferred to semisolid multiplication media containing the same MS medium base (Murashige and Skoog 1962), plus inositol 100 mg L^{-1}, agar 5.5 g L^{-1}, sucrose 30 g L^{-1}, thiamine, nicotinic acid and pyridoxine at 0.5 mg L^{-1}, glycine 2 mg L^{-1}, *Plant Preservative Mixture*® (PPM) at 0,2 g L^{-1} and activated charcoal 5 g L^{-1}. With the same pH and autoclaving procedure used previously.

The induction of callogenesis was carried out in foliar (0.5 x 0.5 cm and lacerated diagonally, 3 times) and stem explants (measured 0.5 cm) of *T. pratense.* They were inoculated into culture medium containing the MS medium (Murashige and Skoog 1962) macronutrients (50 mL L^{-1}), inositol 100 mg L^{-1}, thiamine 0.1 mg L^{-1}, nicotinic acid and pyridoxine at 0.5 mg L^{-1}, casein 1 g L^{-1}, glycine 2 mg L^{-1}, phytagel ® 2 g L^{-1}, sucrose 30 g L^{-1}, pH 5.5.

Three growth regulator (GR) combinations were added to the culture medium, according to previous studies in our laboratory (data not shown): callogenesis induction medium I (CIM-I) - without regulators; CIM-II - naphthalene acetic acid (NAA) 0.1 mg L^{-1} + 6-benzylaminopurine (BA) 0.5 mg L^{-1} and CIM-III - NAA 2.5 mg L^{-1} + kinetin 5 mg L^{-1}. The autoclaving procedure was the same that we used earlier and poured into sterile and disposable Petri dishes. The parameters evaluated were: the percentage of callogenesis (for stem and leaf explants) and the callus friability (Silva 2012) after 30 days of cultivation.

Callus induced in the semi-solid medium were transferred to liquid medium for the development of the suspension cell cultures. Cell suspensions were initiated by transferring 0.5 g of friable callus to 30 mL of liquid medium in Erlenmeyer flasks (125 mL), in a shaker (100 rpm), in the dark, at 25 ± 2 °C. MS medium was used at the concentration of 50% salts. To this were added inositol 50 mg L^{-1}, sucrose 15 g L^{-1},

thiamine, nicotinic acid and pyridoxine at 0.25 mg L^{-1} glycine 0.5 mg L^{-1}, PPM 0.2 g L^{-1}, adenine 50 mg L^{-1}, casein 1 g L^{-1}. The pH was adjusted to 5.5 and autoclaving was performed under the same conditions as above. The medium was changed each four weeks. The enzyme pectinase 0.005% was filter-sterilized and added to half of the cultures after autoclaving the medium (Mustafa *et al*. 2011). This medium was used in the first 4 weeks of culture, the exchange was performed every two weeks and then pectinase was withdrawn in the remaining subcultures until 60 days of culture was completed.

Subcultures were performed by transferring 30 ml of fresh medium into the culture of suspension cells and distributing them in two new flasks. Compared with weighing or pipetting, the liquid transfer technique is simpler (less tools used), faster, with less chances of contamination and stress of the cells in this initial phase of cultivation, but presents low reproducibility (Mustafa *et al*. 2011).

Extraction and chemical analysis of isoflavones

The flavonoids analysis by Ultra Performance in Liquid Chromatography (UPLC) was adapted from the literature (Galland *et al*. 2014) and the samples were compared to standards of the commercially acquired isoflavones daidzein, genistein, formononetin and biochanin A (Sigma-Aldrich®). The cell cultures were filtered to eliminate the medium and after, were lyophilized, the same was done with the plants. The extraction was made using around 100 mg of *T. pratense* plant material, which were reduced to powder using liquid nitrogen mortar and pestle. To this powder we added 1.5 mL of extractive solution (methanol / water / acetone / TFA, V/V 40/ 32/ 28/ 0.05 %), and the membranes were further ruptured by placing the samples in an ultrasonic bath for 20 min at 25kHz at 4°C and then centrifuge, at 20000 g for 20 minutes. The two resulting supernatants were pooled and dried in a rotary evaporator and lyophilizer. The dry pellet was dissolved in acetonitrile (ACN): water (1:1 V/V) and filtered in 22 μm (Millipore®).

UPLC isoflavones quantitative analysis

The extracts of *T. pratense* wild, *in vitro* plants and suspension cultures were analyzed by Acquity® UPLC (Waters Co., MA, USA) equipped with a Waters Photodiode Array Detector eλ UV detector. The separation of the compounds was achieved in a reverse phase (Acquity UPLC ® BEH C18, 1.7 μm, 2.1 x 50mm, Waters, Ireland), using 3 μL of each sample in a flow rate of 0.3 μL /min, at 40 °C and a binary gradient: (A) water and formic acid 0.1% (V/ V) and (B) ACN and formic acid 0.1% (V/ V). The solvent gradient was programmed as follows: 0-2 min 95% A, 2-4 min 90% A, 4-17 min 60% A, 17-21 min 100% B, 21-23 min 100% B. The wavelength analyzed was at 260 nm.

Quantitative analyzes were performed using commercial standard isoflavones to verify the UV absorption, retention time and the linearity. Each curve was made containing six different concentrations each, for daidzein the concentration range used was 1.5-5.5 μg mL^{-1}, for genistein 4.3-216.4 μg mL^{-1}, for formononetin 76.3-225 μg mL^{-1} and for biochanin A 5.9-357.2 μg mL^{-1}. The linear equation and the determination coefficient (R^2) were calculated for each standard isoflavone curve.

Statistical analysis

The germination experiments were completely randomized, composed of 3 different concentrations of culture media and 15 replicates per treatment, considering each plate containing 10 seeds an experimental unit. The callogenesis induction experiments were performed in duplicate, with 17 replicates per tissue and treatment. Cell suspensions were run in duplicates for each cell line. The replicates of each treatment were combined prior to extraction and analysis of isoflavones. The significant differences of each treatment were determined with an analysis of variance followed by the Tukey test at 0.05% probability level using Winstat 1.0 Software (MACHADO and CONCEIÇÃO, 2002). Isoflavone concentrations were determined by means of the standard curves for each one of the isoflavones analyzed in the samples.

Results and discussion

Disinfestation and germination

The employed disinfestation protocol was shown to be quite effective for seeds (Table 1). The highest percentage of contamination occurred in the seeds present in the media with 50% of the MS salts, followed by the medium with 100% and the 25% with zero contamination detected. A percentage of contamination up to 10% demonstrates the efficiency of the disinfestation process and allows to obtain good results of plant micropropagation.

Another important point is that the higher the concentration of active chlorine, the higher the pH of the disinfecting solution (which should be between 5 and 8), and a higher pH favors the development of some bacterial defense mechanisms and even in high concentrations of active chlorine, the action of the product is difficult (Donini *et al*. 2005; Braga *et al*. 2015). But, during our manipulation, it was observed that even with a high concentration of active chlorine (11%), the medium containing 25% of MS salts, sucrose 7.5 g L^{-1} and inositol 25 mg L^{-1}, did not present microbial contamination. In the analyzed samples, being the lower availability of carbohydrates one of the factors responsible for this advent.

Seed germination was evaluated at day 7 and 30, after inoculation (Table 1). One week after to the start of cultivation, some seedlings had already emerged from the seeds (Figure 2). The mean values differed statistically and the best results were found in the media containing 50% and 25% of MS salts, respectively, with a germination percentage of the inoculated seeds in 83.5% and 80%. Studies with murmuru (*Astrocaryum ulei*) were able to prove that at low concentrations of sucrose, good percentages of germinated plants are obtained, due to the fact that many species use the embryo reserves themselves for the emission of the plumule and the radicle, being almost autotrophic and without need for energy source supplementation (Pereira *et al*. 2006; Reis *et al*. 2008).

The addition of components to the culture medium, especially macronutrients and carbon sources, represents a considerable decrease in the osmotic potential of the medium, thus, different availability of water supply will cross the tegument barrier and hydrate the seed to trigger the metabolic process of germination, consequently, the germination takes longer to start, which explains the results found in our study with the medium containing the total salts of MS medium (Koné *et al*. 2015).

For some species like *Pyrostegia venusta* (Braga *et al*. 2015), there is a greater variability of behavior in relation to the water availability. By modifying the concentrations of agar and remaining with the same concentrations of macro and micronutrients during the germination of *Vigna subterranea* (L.) seeds, researchers observed that the success of germination is entirely related to the water availability of the medium (Koné *et al*. 2015).

Table 1. Percentage of germination and contamination in *Trifolium pratense* seeds after 30 days of cultivation

Medium	% contamination	% germination
100% MS	11.5 B	29.0 B*
50% MS	18.5 A	83.5 A
25% MS	0 C	80.0 A

* Averages with different letters, differed statistically according to the Tukey test (0.05%).

Figure 2. Plantlets of *Trifolium pratense* 7 days old (A) and 30 days old (B).

Multiplication, induction of friable callus and initiation of cell culture

In the multiplication media, the addition of activated charcoal was employed due to the fact that the plants presented hyperhydricity (Figure 3), and the situation was reversed with the addition of the charcoal to the medium. It was demonstrated that this component significantly reduces hyperhydricity in some species, for example in shoots of onion crops, however, these results are quite species dependent, for artichoke there was no reversal, and for *Picea abies* and *Sequoia sempervirens* it induced this condition, also, charcoal induce the rooting on media (Pan and Van Staden 1998; Klenotičová *et al*. 2013).

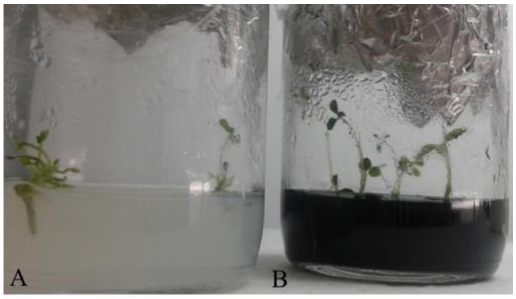

Figure 3. Trifolium pratense in vitro plants, cultivated in MS medium without charcoal, with hyperhydricity **(A)** and in the presence of activated charcoal, healthy **(B)**.

Regarding the stems and leaf explants, 30 days after inoculation in the medium with different concentrations of phytohormones, we evaluated the percentage of callus formation and the callus friability. Thus, it can be observed (Table 2) that in the media without hormones, the callogenesis was practically null in the two evaluated periods. The best results were in the CIM-III medium (NAA and kinetin) both for the induction of callogenesis and friable callus formation. The induction of friable callus did not differ statistically for stems and leaves in this medium.

In studies with callus for the red clover plants regeneration, the media containing a synthetic auxin, such as 2,4-dichlorophenoxyacetic acid (2,4-D) and another auxin (NAA) or cytokinin (kinetin) presented better results using the Gamborg B5 base medium. The composition of this culture medium differs from the MS with respect to nitrogen and phosphorus concentrations (Horvath Beach and Smith 1979; Khanlou *et al*. 2011). In another research, in MS medium, the callus (with regenerative potential) showed a large percentage of formation in media with 2,4-D, using explants of smaller sizes (Kokina *et al*. 2005).

Due to the fact that our investigation seeks the formation of callus that are used in the production of secondary metabolism molecules, they do not need to have the capacity of regeneration as mentioned above, what justifies the choice of the use of a phytohormone that presents results subtler than 2,4-D in the induction of morphogenetic responses in plant tissues, as well as the use of the MS medium, with a related lower cost and easy preparation. As reported, the addition of NAA in the medium may be effective in the formation of phenolic compounds and related molecules in the callus (Ercetin *et al*. 2012).

Table 2. Percentage of callogenesis and friable calli in *Trifolium pratense* explants after 30 days of treatment.

Medium	Hormone levels (mg L^{-1})			% callogenesis	% friable calli	
	NAA	BA	Kinetin		Leaves	Stem
CIM - I	-	-	-	1.64 C	0	0
CIM - II	0.1	6.0	-	28.7 B	0	0
CIM- III	2.5	-	0.5	42.28 A	21.0 A	33.0 A

* Averages with different letters, differed statistically according to the Tukey test (0.05%).

In Figure 4, it is possible to visualize the explants after the 30-day treatment in the CIM-I and CIM-II media. The leaves and stems (Fig. 4*A*; *B* and *C*) that presented some morphological modification, had the formation of hairs with slight darkening in the stem explant. In the CIM-II medium, hair formation and maintenance of the chlorophyll pigments in the leaves (Fig. *4D*) and necrosis in a little part of the leaf explants (Fig. 4*D*), and in the stems (Fig. 4*E* and *F*), with a large browning area, with a big cell formation surrounding the explant.

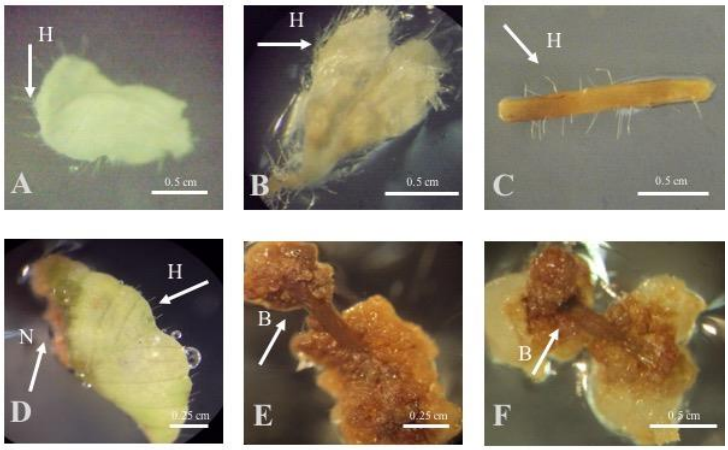

Figure 4. Explants of *Trifolium pratense* grown in different media after 30 days of cultivation. A and B) CIM-I leaves; C) CIM-I stem; D) CIM-II leaves; E and F) CIM-II stem. Abbreviations: H (hair); N (necrosis); B (browning). Scale bars represent 0.5 and 0.25 cm.

The callus formed were homogeneous, with a light-yellow coloration and no regeneration ability under this cultivation conditions. Some studies suggest that there are variants of callus cultures that are no longer able to undergo differentiation in intact plants. However, these cell lines may be useful, even in their undifferentiated form, as in the *in vitro* production of desirable metabolites (Skirvin 1978).

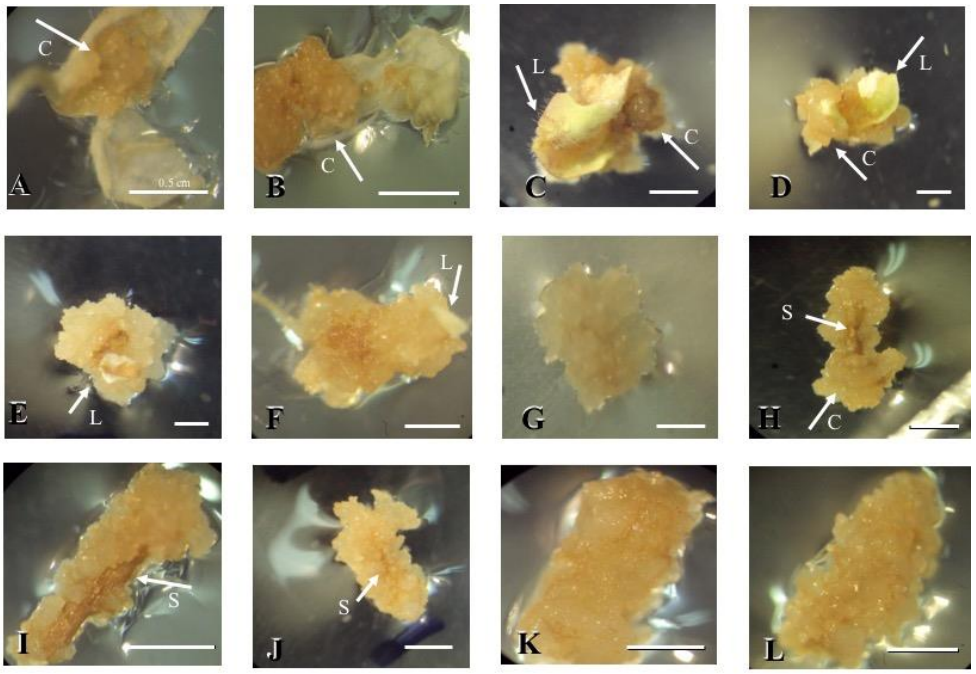

Figure 5. Explants of *Trifolium pratense* grown in MICB-II (photos A and B) and MICB-III (in the others photos), after 30 days of cultivation. Leaf explants: A and B indicate some spots where the cells are covering the explant; C and D shows the cells and the explant; E and F little piece of the leaf explant; G, only the callus can be visualized, with the explant totally hidden by the cells. Stem explants: H demonstrate the cells appearing on the sides and covering the explant; I part of the explant can still be visualized; J indicate a large part of the cover explant, only the central part with a small piece of the explant; K and L, only the callus, with the caulinar explant totally hidden by the cells. Abbreviation: C (cell formation); L (leaf explant); S (stem explant). Scale bars represent 0.5 cm.

In the callus formation, it can be seen that the cell growth occurred in the central part of the leaf explants (Fig. 5*A* and *B*) when the explants were cultivated in CIM-II medium; in the case of CIM-III medium (Fig. 5*C* and *D*), the number of cells increased and covered the explant (Fig 5*E* and F) until the leaf explant was taken up by the cells (Fig. 5*G*). The cell formation in *T. pratense* stem explants (CIM-III medium) started at the ends of the explant (Fig. 5 *H* and *I*) and expanded throughout the whole explant, hiding the stem (Fig. 5*J*), the explant can be seen totally covered by the cells in Fig. 5*K* and 5*L*. Callus from leaf explants took longer to present the same size as stem callus in the end of the 30 days.

It is known that increasing auxin levels generally promotes an intensification in friability and a reduction in cell differentiation, this is due to the fact that friability has a tendency for the cells to round off and separate just after division , promoting a rupture

of the cell-cell contacts, which could contribute to the differentiation mediated by neighboring cells (Williams *et al*. 1990).

Similar results were found with callus from leaves, epicotyl and cotyledonary explants from *T. pratense* grown in MS and PC-L2 medium. This cell formations present different colors, and the most friable were those of yellow coloration, and the callogenesis in medium PC-L2 was faster. Reports show that the addition of NAA to the culture medium can be quite effective in the production of phenolic compounds and their correlates in callus (Çölgeçen and Toker 2008; Ercetin *et al*. 2012).

Figure 6. (A) Culture media after the callus of *Trifolium pratense* after transfer of callus to the onset of suspended cell cultures; (B) cell suspensions after 30 days in rotary shaker; (C) suspension cultures, without enzyme added to the medium (left photo) and 30 days after the addition of the pectinase (photo in the right) to aid in the release of the cells in the medium.

From the 45th day in culture, the friable callus formed were transferred to the liquid culture media (Fig. 6*A*) under agitation, and the cultures of the cells were suspended (Fig. 6*B*). According to the different callus that originated them, we obtained distinctive cell cultures, with diverse colorations (Fig. 6*B*). In the Fig. 6*C*, we can observe the variance between the medium without and with pectinase, this enzyme can break down the pectin in the cell wall, releasing cells more easily than just by shaking them in the medium. Cell cultures in *T. pratense* (tetraploid variety) were previously performed using the Gamborg medium, with the addition of 2,4-D and BA and with culture intervals similar to those performed in our findings (Kašparová *et al*. 2006; Kašparová *et al*. 2012). After all this process, we proceed to the chemical analysis of the content, searching for the standards selected by us.

Extraction and chemical analysis of isoflavones

T. pratense is a rich source of isoflavones and has become the focus of several studies related to its phytoestrogenic activity. Based on the fact that the native forms of isoflavones (glycosides and malonylglycosides) can be affected by several factors, such as seasonality, culture, harvest and storage, the analyzes of the present work were carried out with the aglycone and the *O*-conjugated forms of the isoflavones. However, the quantification was performed with commercial standards of the four isoflavones most studied and identified in the samples according to the retention time of this molecules.

The calculations to determine isoflavonoid concentration in the wild clover and *in vitro* cultured samples after 30, 45 and 60 days of cultivation were performed using the linear equations for each standard isoflavone curve: daidzein: y=5266,7x + 1741,7 (R^2 = 0.9974); genistein: y = 15264x – 47994 (R^2 = 0.99956); formononetin: y = 6068,4x + 43924 (R^2 = 0.99788) and biochanin A: y = 7802,7x + 22006 (R^2 = 0.9998).

The limits of detection (LOD) were stablished at a signal-to-noise ratio (S/N) of 3 and the limits of quantification (LOQ) at the signal-to-noise ratio (S/N) of 10. The LOD and LOQ were calculated for each isoflavone, in this way, for daidzein (0,19 and 0,58 μg mL^{-1}), genistein (0,69 and 2,08 μg mL^{-1}), formononetin (4,73 and 14,34 μg mL^{-1}) and biochanin A (2,94 and 8,90 μg mL^{-1}).

According the analysis of the standards in the method used in this study (Galland *et al*. 2014), were detected the isoflavones: daidzein (retention time 10.598 min), genistein (12.648 min), formononetin (14.744 min) and biochanin A (17.541 min) with their characteristic UV spectra. Due to the variability presented in the production of the various types of isoflavones in this species, the initial analysis of the plants was performed to determine the existence of this molecules in the red clover wild (from the field), *in vitro* plants and in the cell cultures cultivated by us.

UPLC isoflavones quantitative analysis

The chromatograms corresponding to the *T. pratense* var. URS-BRS Mesclador (wild) and *in vitro* grown plants presented different characteristics, and the chromatographic peak relative to daidzein and genistein was not visualized in one sample (Table 3). The presence of constant fluctuation between the concentration of the various types of isoflavones content (Spagnuolo *et al*. 2014), leads us to believe that the

growing conditions in which these plants were harvested did not provide the favorable metabolic balance for the biosynthesis of these specific molecules.

Table 3. Isoflavone quantification in wild and *in vitro* cultivated plants of *Trifolium pratense* (mg g^{-1} dry material) ± standard deviation.

Samples	**Daidzein** y = 5266,7x + 1741,7	**Genistein** y = 15264x - 47994	**Formononetin** y = 6068,4x + 43924	**Biochanin A** y = 7802,7x + 22006	**Total isoflavone content**
In vitro – 30 d	0.229 ± 0.012	0.173 ± 0.0002	9.086 ± 0.144	0.585 ± 0.053	10.074 ± 0.065
In vitro – 45 d	1.361 ± 0.030	0.419 ± 0.1982	8.513 ± 0.100	1.805 ± 0.763	12.098 ± 0.334
In vitro – 60 d	17.590 ± 0.213	0.113 ± 0.0070	3.774 ± 0.319	2.055 ± 0.189	23,532 ± 0.129
Wild (1)	0.364 ± 0.0	0.193 ± 0.0295	15.564 ± 1.113	0.781 ± 0.113	16,903 ± 0.535
Wild (2)	*	*	12.689 ± 0.073	1.849 ± 0.073	14,539 ± 0.042

* Trace or peak not identified in the sample. Abbreviations: d (days in culture).

The difference between this two wild samples are the months of harvest, in different seasons of the year, although the collection site and the cultivar were the same. The sample Wild 1 was collected in 2015/ May and, the sample 2 in 2015/ December. In the South Hemisphere, this period of time represents Fall (May) and Summer (December). Through the website of the National Institute of Meteorology of Brazil (Ministério da Agricultura Pecuária e Abastecimento 2015) we were able to visualize changes in the temperatures referring to the months in which the plants were harvested (Table 4), showing us differences related to daily temperatures, hours of sunshine and even the amount of rain that these plants received during these different months/seasons of the year.

Table 4. Average temperatures, sunlight and rainfall daily in the months of collection of the wild plants of *Trifolium pratense.*

Harvest	Daily temperature (℃)		Total daily sunlight (hours)	Rainfall (mm)
	Maximum	Minimum		
May/2015	23	13	5.24	140
December/2015	29	17	7	100

Source: INMET/2015

According with this information (Table 4), we know that the plants collected in May, passed through periods of lower temperatures, less daily hours of sun and rainfall higher than the plants collected in the month of December and consequently in the

Brazilian summer. In United States, researchers found that the isoflavones of red clover flowers are produced better at high temperatures and for the above-ground parts of the plant, they present greater biosynthesis when there is a higher incidence of rainfall (Booth *et al*. 2006). Similar results were find with soybean (*Glycine max* [L.] Merr.) isoflavones, were the content of daidzein and genistein were higher with irrigation, agreeing with our results, which presented maximum isoflavone content when the plants were collected in the coldest and rainy season analyzed (Bennett *et al*. 2004).

The isoflavonoid that presented the highest concentration in the wild-red clover samples was formononetin (15.564 and 12.689 mg g^{-1}) and, in smaller quantities, biochanin A (1.849 and 0.781 mg g^{-1}), daidzein (0.364 mg g^{-1}) and genistein (0.193 mg g^{-1}). Slightly different results were reported in *T. pratense* flowers, in which formononetin was the major isoflavones, followed by genistein and biochanin A and a very small amount of daidzein (Ercetin *et al*. 2012). In another study, comparing the content of different organs in wild plants, the standards were detected in all the organs, except daidzein which was not found in flowers, also, daidzein and genistein were detected in lower concentrations than the other standards (Saviranta *et al*. 2008).

As shown in Table 3, in this wild samples evaluated, it appears that the metabolism is directed to the biosynthesis of formononetin, with high productivity and secondly to the biochanin A. Biosynthetically, there is a possibility of bioconversion using the 4'*O*-demethylation of biochanin A and formononetin, to form genistein and daidzein (Fig.1), respectively. In agreement with our data, a survey that assessed the content of isoflavones different organs of *T. pratense*, determined that biochanin A and formononetine were in greater concentration and the organ with the highest productivity of these molecules were the leaves (Wu *et al*. 2003).

With respect to the contents present in the *in vitro* plants grown (Table 3), those grown after 60 days presented the largest increment detected, corresponded to the isoflavonoid daidzein (17.590 mg g^{-1}), after which, the following concentrations were formononetin at 30 days (9.086 mg g^{-1}) and 45 days (8.513 mg g^{-1}) as well as at 60 days (3.774 mg g^{-1}). Subsequently, the biochanin was detected in plants with 60 (2.055 mg g^{-1}) and 45 days (1.805 mg g^{-1}) and, finally, daidzein in plants with 45 days of *in vitro*

culture (1.361 mg g^{-1}). For this cultivar, genistein had the lowest productivity detected, of the four quantified isoflavones.

Thus, chronologically, in the plants with 30 days of culture, formononetina started with the highest levels, and to a lesser extent, biochanin A, genistein and daidzein, with the lowest concentration detected (Table 3). The same pattern was respected in the plants at 45 days of cultivation, but the arrangement was modified in the plant analyzes with 60 days, where the concentration of daidzein increased, becoming the most produced and the formononetin, biochanin A and daidzein.

This fact demonstrates that even under laboratory conditions, there is a change in the biosynthesis patterns of these molecules, suggesting the need for long-term studies to better examine these changes. Studies with callus of *T. pratense* report that the concentration of some molecules decreases from the third subculture, most of the time, agreeing with our results, however, an increase in the concentration of formononetin and genistein is usually detected, differing from what occurred in our plants (Ercetin *et al.* 2012). Studies prove that some hypocotyl lesions can cause a dramatic increase in the level of daidzein in soybean leaves (Morris *et al.* 1991), what may happened during the subcultures, when you manipulate the plants to change to new medium.

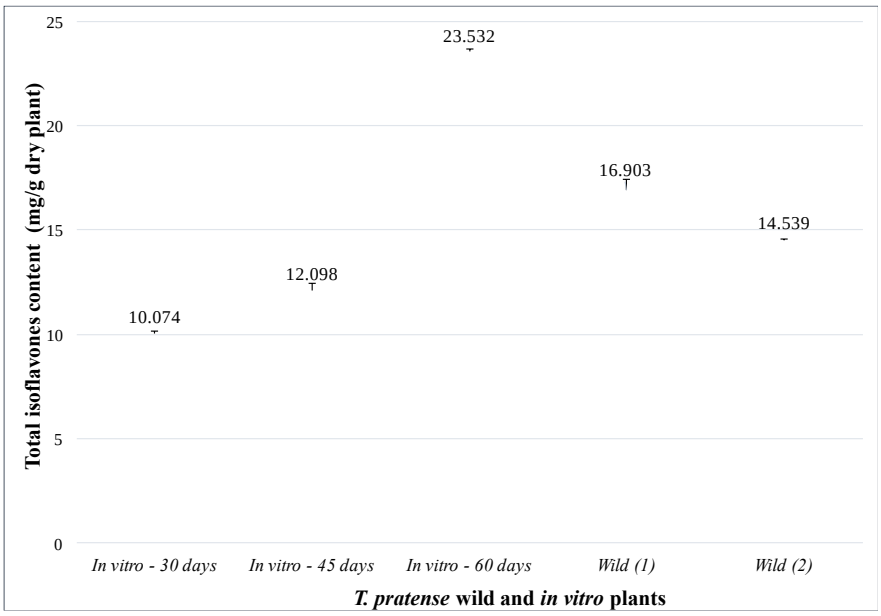

Figure 7. Total isoflavone content (mg g^{-1} dry plant) in *Trifolium pratense* plants wild and cultivated *in vitro* after 60 days.

Comparing the total isoflavone content of the wild and *in vitro* samples (Table 3; Fig. 7), we can visualize an increase in the concentration of isoflavones over time in the plants grown *in vitro* and in the wild plants, those collected in winter (wild 1) presented a higher total concentration. Our data *in vitro* at 60 days is similar to the total contents found in red clover leaves analyzed by Tsao *et al*. (Tsao *et al*. 2006) containing 23.43 mg g^{-1} dry material, results in higher concentrations than others already published (Vetter 1995; De Rijke *et al*. 2001; Wu *et al*. 2003; Sivesind and Seguin 2005). The fact that *in vitro* plants have a higher concentration, even without the elicitation involved in the process, may be due to the modification in the culture process that occurs and to the stress that the plants are exposed when they grow inside of an *in vitro* vessel (Georgiev *et al*. 2010).

The callus were induced, using the aerial part of the plant, which, as previously reported, has the highest total of isoflavones, thus, the cell culture can be a good alternative, since it has acilitated and quicker multiplication than tissue and organ culture, with a very large industrial potential, and can be grown on a large scale, as in bioreactors (Naaz *et al*. 2014). After obtaining the callus with the desired characteristics, seeking to obtain homogeneous cultures, we used pectinase to reduce clumps and to produce a homogeneous culture. The pectinase can disturb the cell walls exterior, but, in a low concentration can break only the aggregates by dilution of the pectins (Mustafa *et al*. 2011). Also, because of the formation of cell wall fragments, this enzymes can act as elicitors (Verpoorte *et al*. 1999), considering this, we used only in a half of the cultures (samples with the "*E*" in the name of the line).

Table 5. Isoflavone quantification in cell culture lineages of *Trifolium pratense* (mg g^{-1} dry material) ± standard deviation.

Samples	Daidzein	Daidzein (%)	Genistein	Genistein (%)	Formononetin	Formononetin (%)	Total isoflavone content
Tp 1	0.13 ± 0.01	33,9	0.25 ± 0.002	66,1	*	0,0	0.38 ± 0.01
Tp 2	0.05 ± 0.01	18,7	0.24 ± 0.001	81,3	*	0,0	0.30 ± 0.01
Tp 3	0.11 ± 0.00	52,3	0.10 ± 0.01	47,7	*	0,0	0.22 ± 0.004
Tp 4	0.02 ± 0.00	5,6	0.11 ± 0.03	34,8	0.19 ± 0.016	59,6	0.06 ± 0.014
Tp 5	0.04 ± 0.01	15,8	0.10 ± 0.004	38,0	0.13 ± 0.01	46,2	0.19 ± 0.001
Tp 6	0.22 ± 0.02	72,7	0.08 ± 0.004	27,3	*	0,0	0.89 ± 0.013
Tp 1 E	*	0,0	*	0,0	0.91 ± 0.012	100,0	0.91 ± 0.01
Tp 2 E	0.11 ± 0.02	12,2	0.13 ± 0.02	14,1	0.67 ± 0.037	73,7	0.91 ± 0.01

Tp 3 E	0.06 ± 0.02	37,2	0.11 ± 0.0004	62,8	*	0,0	0.17 ± 0.01
Tp 4 E	0.06 ± 0.00	100,0	*	0,0	*	0,0	0.32 ± 0.001
Tp 5 E	0.05 ± 0.01	25,5	0.14 ± 0.001	74,5	*	0,0	0.28 ± 0.006
Tp 6 E	0.61 ± 0.01	68,6	0.28 ± 0.004	31,4	*	0,0	0.30 ± 0.01

* Trace or peak not identified in the sample.

In the analyzes performed with the cell cultures (Table 5), there was no detection of the isoflavone biochanin A in any of the cultures performed, it was observed that some cultures presented only one of the analyzed standards, like for *Tp1E* lineage (formononetin) and *Tp4E* (daidzein), or even the absence of some of the analyzed isoflavones, as occurred with the *Tp1*, *Tp2*, *Tp3*, *Tp6*, *Tp3E*, *Tp5E* and *Tp6E* lines that did not present formononetin. Others produced the three compounds (*Tp4*, *Tp5* and *Tp2E*). The highest productivity for two of the molecules was found in the *Tp6E* line, which presented the highest levels of daidzein and genistein detected in the cultures, 0.6090 and 0.2793 mg g^{-1} of dry cells, respectively. In *Tp1E* only formononetin (0.9071 mg g^{-1}) was found, but with the highest levels identified.

Isoflavonoids content were also examined with the culture medium where the cells were cultured, but we could not find detectable levels of this molecules, in none of the samples that we analyzed. Thus, we can say that in the cell lines produced in our experiment, there was no transference of the analyzed molecules from the plant cells to the culture medium or they are properly degraded.

About the total isoflavone content (Table 5; Figure 8), three cell lines presented the total content superior to the others, two of them, with the addition of pectinase during the initial part of the culture, *Tp1E* and *Tp2E* and one without, *Tp6*. The total contents of the three were very similar, but if we see the composition of the patterns, the *Tp6* line is composed by daidzein (73%) and genistein (27%); *Tp1E* sample was composed only by formononetin (100%) and the *Tp2E* line was composed of three of the standards: daidzein (12%), genistein (14%) and formononetin (74%).

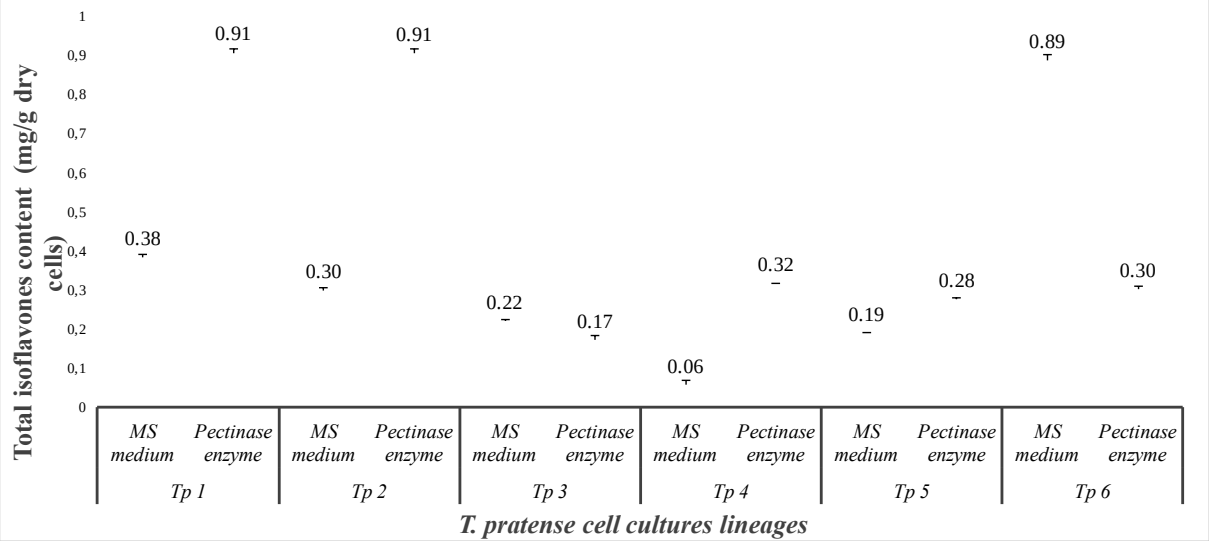

Figure 8. Total isoflavone content (mg g^{-1} dry cells) in *Trifolium pratense* different lineages of cell cultures.

For the comparison between lines (with or without the pectinase) (Fig. 8), four lines containing the enzyme presented higher isoflavones content (*Tp1E*, *Tp2E*, *Tp4E* and *Tp5E*). The other two had better result only with the control, in *Tp3*, there was little difference between them (*Tp3*≥ *Tp3E*); and for *Tp6*, the line without the enzyme presented higher productivity than the other (*Tp6E*).

In a study with *Lithospermum erythrorhizon*, *Polygonum tinctorium* and *Solanum melongena* cells, the use of pectinase was effective for increasing the yields of secondary metabolites (Imoto and Ohta 1988; Hiroshi *et al*. 1990; Ju Hwan *et al*. 1997). In strawberry cell cultures with the use of pectinase, the cultures produce anthocyanins and other phenylpropanoid metabolites (Edahiro and Seki 2006), presenting a good correlation between cell-aggregate formation and secondary metabolism, also, they suggest that the size of the cells can alter the cell accumulations of this metabolites. As shown in Fig. 6-*C*, studies with *Nicotiana tabacum* cells also demonstrated browning by adding pectinase to the culture medium (Negrel and Javelle 1995) and the use of pectinase also induce the phenylpropanoid pathway in this cultures.

Even isoflavone-producing cell cultures may not produce one of the phytoestrogens present in the plants that gave rise to them, as is the case of *T. pratense* cultures in which the presence of genistein was not observed. Studies related to the production of phytoestrogens in cell cultures of *Psoralea corylifolia* and *T. pratense* have been reported that their induction is dependent on cell growth and plant hormone

elicitation, such as IAA (3-indoleacetic acid), NAA (1-naphthaleneacetic acid), kinetin or 2,4-D (2,4-dichlorophenoxyacetic acid) (Çölgeçen *et al.* 2014; Satdive *et al.* 2015).

With respect to the isoflavones composing the lineages (Table 5; Figure 10), some cultures did not present difference when the pectinase was added in the culture medium, like *Tp3* and *Tp3E*; *Tp6* and *Tp6E*, however, the percentage that composes them was slightly altered, as can be seen in Table 5. The others present modifications for the types of isoflavonoids that make up (*Tp1* and *Tp1E*); by the biosynthesis of a molecule previously absent in the composition (*Tp2* and *Tp2E*); or even by the negative regulation of the biosynthesis of one or more of them (*Tp4* and *Tp4E*, *Tp5* and *Tp5E*).

We believe that the differences in the extract compositions may occur due to the fact that cell wall degradation enzymes can induce a wide range of defense responses, inducing the biosynthesis of different metabolites, even in the same cell lines (Negrel and Javelle 1995). Also, there are reports in which the metabolism of the cells can be modified when in culture. For many pharmaceutical compounds, production in cell cultures is close to zero due to the fact that they originate from undifferentiated cells which leads many of the metabolites not to be formed by the need for tissue-specific biosynthesis. It is demonstrated that sequential biosynthetic enzymes can occur in different cell types, with the need to translocate route intermediates between cells, emphasizing the complex cellular biology necessary in the biosynthesis of these molecules (Facchini 2001; Ziegler and Facchini 2008).

Conclusion

The *in vitro* cultures showed a production of the isoflavonoids with modifications in the course of the subcultures, which confirms that even under controlled conditions, there are modifications in the metabolism of the cultures, being necessary long term studies to realize the kinetic curve of the isoflavones present in the plants this variety. The wild plants analyzed showed a higher content of isoflavones when grown in cold and rainy climates.

It is possible to conclude that the best medium for the formation of friable calli was the CIM-III medium, with callus formation quality and great applicability for the initiation of cell cultures with *Trifolium pratense* leaves and stem. All cell cultures

biosynthesized isoflavonoids, with different compositions and concentrations, and isoflavone profile different from that found in wild plants, allowing the discovery of different lineages for the production of molecules of commercial interest for a certain biological activity.

Thus, our study provides important information for posterior studies with the red clover as a source of nutraceuticals, functional foods and phytomedications with rich content of isoflavones.

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CAPÍTULO V

Isoflavone content in hairy root cultures and plantlets of *Trifolium pratense*

1. Introduction

Legumes have an agricultural and economic importance rather large, are consumed in great quantities by humans and animals due to their nutritional value (FARAG *et al*., 2007), and for production of phytoestrogenic compounds. These compounds exhibit some similarities with the structure and the molecular weight of 17β-estradiol (Fig. 1-*e*) and, this way, bind with the ligand binding pocket with the receptor of estrogen being able to act as agonists or antagonists, with action determined in a dose-dependent manner (BECK; ROHR; JUNGBAUER, 2005; CHAN; WANG; LEUNG, 2003).

Isoflavonoids are a distinct class among flavonoids originated from the phenylpropanoid pathway (Fig. 1) and can be divided into isoflavones and pterocarpan (medicarpin) (SAVIRANTA *et al*., 2010). The majority molecules of isoflavones are formed by a split route, according to their key intermediates, genistein originates through naringenin and subsequently, via isoflavone 4'-*O*-methyltransferase enzyme can generate the biochanin A. For daidzein, the intermediate is liquiritigenin and with the action of the same isoflavone 4'-*O*-methyltransferase, formononetin is derived (DU; HUANG; TANG, 2010; KANEHISA *et al*., 2017).

Red clover (*Trifolium pratense* L.) is a forage legume that is among the most cultivated in the world because of the rapid growth and soil improving characteristics (BECK; ROHR; JUNGBAUER, 2005; DIAS *et al*., 2008) and since the 1950s was determined and identified as accumulating estrogenically active compounds, first in sheep (BENNETTS; UNDERWOOD; SHIER, 1946) and later in humans the main isoflavones present in this species are formononetin, biochanin A, daidzein and genistein (Fig.1-*a* to *d*) (SAVIRANTA *et al*., 2008) and these molecules have proven use in relieving menopausal symptoms (VAN DE WEIJER; BARENTSEN, 2002), opiate activity (NISSAN *et al*., 2007), preventing osteoporosis (OCCHIUTO *et al*., 2007), estrogenic effects (JOUNG; KIM; SHEEN, 2003), prostatic cancer (JARRED *et al*.,

2002), hormone replacement therapy (BECK; ROHR; JUNGBAUER, 2005), lowering LDL cholesterol (NESTEL *et al.*, 2004) and anti-inflammatory diseases (CEOLATO; SCHAPOVAL; AGNOL, 2012).

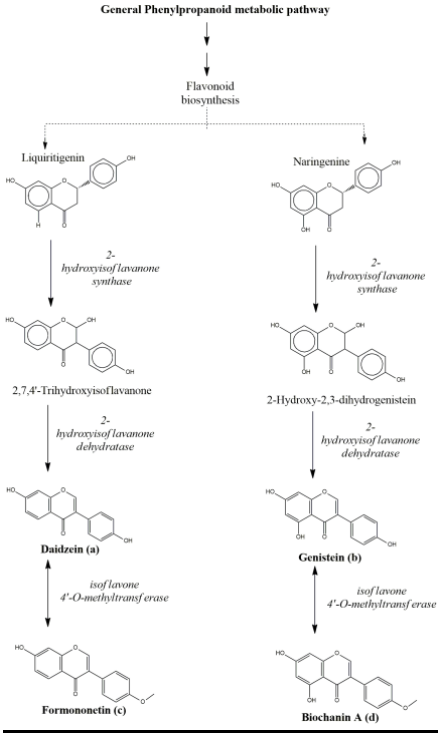

Figure 1. Synthesized biosynthetic route of isoflavonoids showing the generally phenylpropanoid metabolic pathway and the isoflavone pathway with the most known molecules: daidzein (**a**), genistein (**b**), formononetin (**c**), biochanin A (**d**) and the estrogen 17β-estradiol (**e**) for molecular comparison. Adapted from (DU; HUANG; TANG, 2010; KANEHISA *et al*., 2017). Abbreviations: PAL phenylalanine ammonialyase, C4H cinnamate-4-hydroxylase, 4CL 4-coumarate CoA ligase.

Many plant secondary metabolites of interest accumulate in roots. Studies done with *T. pratense* roots reported that the concentration of aglycone isoflavones was 39 times higher in the potted plant roots compared to the field plant roots, and the roots are the organ with the greatest abundance of the majority isoflavones (SAVIRANTA *et al.*, 2010). However, the harvesting of these organs ends up destroying the plant, a good alternative for facing this problem is the development of transformed roots called hairy root cultures (GUILLON *et al.*, 2006).

The genetic manipulation of the plant roots using as gene vector *Agrobacterium rhizogenes* can offer a convenient technique to produce red clover root cultures with isoflavone biosynthetic potential. The bacterial genes transferred into the plant induce growth of characteristic hairy roots (HR) in the place of the infection, in culture, these

roots are cytologically stable, exhibit continued growth without any supplement, have negative gravitropism and production of opines. This stability allows the biochemical and genetic manipulation in this type of culture (WEBB *et al*., 1990) and the development of controlled cultures, diverging from field crops, which deals with the fluctuation in individual isoflavone content (SPAGNUOLO *et al*., 2014). To date, two studies have carried out the transformation of axillary roots into red clover plants (BEACH; GRESSHOFF, 1986; WEBB *et al*., 1990), but none of them had as objective the production of secondary metabolites.

Plants produce various natural products as part of the development process or in response to the assorted environmental stresses like biotic and abiotic elicitors, ultraviolet light, plant hormones and fungicides and many secondary metabolites that are in traces or low amounts in a healthy tissue are synthesized in high quantities in response to this traumas (KAIMOYO *et al*., 2008; TEBAYASHI; ISHIHARA; IWAMURA, 2001). Therefore, elicitation is an efficient way to increase the production of molecules of interest in HR (GUILLON *et al*., 2006), as with *Psoralea corylifolia* (SHINDE; MALPATHAK; FULZELE, 2010) which raised the levels of daidzein and genistein in the presence of low concentrations of phosphate or with *Pueraria candollei* (UDOMSUK *et al*., 2011) were all the elicitors stimulated the production of isoflavonoids.

The aim of our study was to develop a *T. pratense* hairy root protocol and select lineages with high isoflavone yield after the optimization of the elicitors with less toxicity and better root length inductors in the plantlets. To our knowledge no information on hairy root cultures of *T. pratense* using elicitation studies on isoflavone accumulation was previously available. Thus, we report a medium term study with the fluctuations of daidzein, genistein, formononetin and biochanin A in the root crops during the first five months of cultivation and the changes in the yield after use sucrose and salicylic acid provided in different concentrations.

2. Experimental

2.1. Plant material

Seeds of *T. pratense* var. URS-BRS Mesclador were donated by Dr. Miguel Dall'Agnol (Faculty of Agronomy - UFRGS). The specimen was deposited in the Herbarium Alarich Rudolf Holger Schultz-HAS (Museum of Natural Sciences - Zoobotanic Foundation of Rio Grande do Sul) registration code HAS 87114, number 4291 (10/14/1986), and the harvest point was determined in the latitude: -30.0331 and longitude: -51.23 [err: ± 29946 WGS84], by the Faculty of Agronomy (UFRGS), Brazil.

2.2. Seed disinfection and germination

In order to initiate the *in vitro* germination process, *T. pratense* seeds were scarified with fine sandpaper and started the disinfection process with sodium hypochlorite (2% active chlorine) plus tween 20 (two drops/ 100 mL) under stirring, for 30 minutes, followed by triple wash with sterile water and inoculation in agar/ water medium (agar 8 g L^{-1}). The seeds were maintained for 48 hours at 4 ° C and transferred to 24 °C (2 days) in the dark. The seedlings start to growth at this point (± 1,5 cm) and are shifted to a growth chamber with 16 h photoperiod (200 µE) in ½ MS medium (MURASHIGE; SKOOG, 1962) Duchefa ® plus sucrose 10 g L^{-1} and agar 7 g L^{-1} (Supplementary material) .

2.3. Screening of elicitors in the roots of T. pratense plantlets

In order to visualize the ideal concentrations and elicitors, the experiment started with *T. pratense* 5 days-old seedlings, being cultivated in the same conditions described above. The elicitation effect was measured at 0, 12, 24, 36, 48 and 72, 96, 120 or 168h after inoculation.

2.4. Elicitor preparation

Elicitation proceeded with Flagellin 22 (Flg22), yeast extract (YE), salicylic acid (SA) and sucrose. Flg22 was used for the experiments with the plantlets roots length in a range 0; 0.25; 0.5; 1 to 2.5 µM (MILLET *et al*., 2010). The YE was tested at 0.5, 1, 2, 3 and 4 g L^{-1} of distilled water, filter sterilized and added after the medium sterilization (SIVESIND; SEGUIN, 2006). For elicitation, SA was dissolved in distilled water and autoclaved at 121 ◦C for 15 min. After sterilization, SA was added at concentrations of 0, 0.01, 0.1, 0.5 and 1.0 mM or 0, 1.38, 13.81, 69.06; 138.12 mg L^{-1}. Sucrose (Sigma Aldrich, ≥99.5% purity) was employed from 10, 30, 60, 90 until 180 g L^{-1}, being

autoclaved with the medium for sterilization and used with agar 7 g L^{-1} or Phytagel™ 2 g L^{-1} as gelling agents to test the influences in the plantlets.

2.5. Hairy root culture

a) *Agrobacterium* culture and tissue infection

The bacterial cells, strain A4TC24 (PETIT *et al*., 1983), were cultured in the flask containing 4 ml of YEB liquid medium + rifampicin 100 µg mL^{-1} and kanamycin 100 µg mL^{-1} for the selection. The incubation occurs at 30°C, in a rotary shaker at 200 rpm. After, one day before the transformation, transfer 100 μL^{-1} of the liquid culture for a YEB agar plate with the antibiotics in the same concentrations. Culture was incubated overnight at 30°C to obtain the layer of *A. rhizogenes* on the plate to proceed with the transformation.

The 7 days-old seedlings were infected aseptically cutting the root meristem and dipping into the *Agrobacterium* layer or wounding the principal root using *Agrobacterium*-filled syringes in the tip of the primary root of the hypocotyls. Inoculated seedlings were kept for two days in ½ MS covering the roots putting the plate in the angle of 45°. After this time, transfer to ½ MS + 400 µg mL^{-1}Augmentin® + 1% sucrose for 15-21 days (photon flux of 40 µmol m^{-2} s^{-1}), 25 ± 2 º C, with the roots convered to remain in the dark (WRIGHT; WANG, 2015).

When the fresh growing HR reached the size of 10-15 cm, they were cut and transferred to new medium, with the same components and subcultured each 30 days. These HR were transferred to the flask containing 50 ml of ½ MS liquid medium, and maintained at 20°C on a shaker (100 rpm), in the dark.

b) Evaluation of the isoflavone content in HR

During the growth and stabilization of the HR cultures, we developed the growth of thirteen lineages, they were harvested each 30 days of culture and the material was stored in -80 °C for posterior flavonoids extraction and control of isoflavone content during this first 5 months of culture.

c) Extraction and chemical analysis of isoflavones

The flavonoids analysis by Ultra Performance in Liquid Chromatography (UPLC) was adapted from the literature (GALLAND *et al*., 2014) and the samples were

compared to standards of the commercially acquired isoflavones daidzein, genistein, formononetin and biochanin A (Sigma-Aldrich®). The hairy root cultures were lyophilized, and the extraction was made using around 100 mg of *T. pratense* samples, which were reduced to powder using liquid nitrogen mortar and pestle. To this powder it was added 1,5 mL of extractive solution (methanol / water / acetone / TFA, V/V 40/ 32/ 28/ 0.05 %), and the membranes were further ruptured by placing the samples in an ultrasonic bath for 20 min at 25kHz at 4°C and centrifuging at 20000 g for 20 minutes. The two resulting supernatants were pooled and dry in a rotary evaporator and freeze dryer. The dry pellet was dissolved in acetonitrile (ACN): water (1:1 V/V) and filtered in 22 μm (Millipore®).

d) UPLC isoflavones quantitative analysis

The extracts of *T. pratense* hairy root cultures were analyzed by Acquity® UPLC (Waters Co., MA, USA) equipped with a Waters Photodiode Array Detector eλ UV detector. The separation of the compounds was achieved in a reverse phase (Acquity UPLC ® BEH C18, 1.7 μm, 2.1 x 50mm, Waters, Ireland), using 3 μL of each sample in a flow rate of 0.3 μL /min, at 40 °C and a binary gradient: (A) water and formic acid 0.1% (V/ V) and (B) ACN and formic acid 0.1% (V/ V). The solvent gradient was programmed as follows: 0-2 min 95% A, 2-4 min 90% A, 4-17 min 60% A, 17-21 min 100% B, 21-23 min 100% B. The wavelength analyzed was at 260 nm.

Quantitative analyzes were performed using commercial standard isoflavones to verify the UV absorption, retention time and the linearity. Each curve was made containing six different concentrations each, for daidzein the concentration range used was 1.5-5.5 μg mL^{-1}, for genistein 4.3-216.4 μg mL^{-1}, for formononetin 76.3-225 μg mL^{-1} and for biochanin A 5.9-357.2 μg mL^{-1}. The linear equation and the determination coefficient (R^2) were calculated for each standard isoflavone curve.

e) Isoflavone identification

Flavonoid extraction was adapted from the literature (KIM *et al.*, 2008), HR flavonoids were extracted from 100 mg of 13 different lineages of *T. pratense* HR cultivated for 60 days, were grounded in liquid nitrogen using mortar and pestle. Then, 1ml of methanol 80% (v/v) was added to the hairy root powder samples and membranes

were further ruptured by placing the samples in an ultrasonic bath for 20min at 25kHz at 4°C (TI-H-5, Elma Hans Schmidbauer GmbH & Co., Singen, Germany) and then on a rotating wheel (MiniLabroller, Dominique DUTSCHER SAS, Brumath, France) for 1h.

After centrifugation during 10min at 20,000g at 4°C, the pellet was extracted further with 1ml of methanol 80% (v/v) overnight at 4°C on a rotating wheel. The two resulting supernatants were pooled, filtered at 0.45µm (0.45µm Millipore PTFE membrane, EMD Millipore Corporation, Billerica, MA, USA) and dried on a SpeedVac for 8h (Savant SPD131DDA, Thermo Fisher Scientific, Waltham, MA, USA). The dry pellet was dissolved in methanol/water/acetone/trifluoroacetic acid (40/32/28/0.05, v/v) and we added 20ng of rhamnetin (Extrasynthese, Genay, France) as the internal standard. Finally, the extract was filtered again (Paper filter grade GF/A, Whatman International Ltd, Maidstone, UK). The extracts were then analyzed by HPLC-electrospray-MSMS (HPLC-ESI-MS/MS).

The compounds were introduced in the ESI source using a Waters 2695 separation module (Alliance, Waters, Milford, MA, USA) equipped with a Waters 2487 dual UV detector. Separation was achieved on a reverse-phase column (Uptisphere C18 ODB, 150*2.1 mm, Interchim, Montluçon, France) using a flow rate of 0.20 ml/min and a binary gradient: (A) acetic acid 0.1% (v/v) and (B) acetic acid 0.5% (v/v) in acetonitrile. The solvent gradient was programmed as following: 0–5 min 86% A, 5-31 min 45% A, 31–41 min 100% B, 41-48 min 86% A (Kerhoas *et al.*, 2006).

Analyses were performed on a Waters Quattro LC triple quadripole mass spectrometer (Waters) operating in MS full scan for the semi quantification and different scanning modes for the identification of the compounds (ESI positive and negative modes). Relevant instrumental parameters were set as following: capillary 2.70 kV, extractor 3V, source block and desolvation gas temperatures 120°C and 350°C, respectively. Nitrogen gas was used to assist nebulization and desolvation (78L/h and 272L/h, respectively). For the MS/MS mode (for the confirmation of the identification: fragmentation “Daughter mode” and molecular ions “Parent mode”), argon gas was used as the collision gas at 2.83µbar (GALLAND *et al.*, 2014).

2.6. Hairy root elicitation

After stabilization of the culture for 6 months, they were elicited. Thus, hairy root cultures with approximately 7 days after subculture were transferred to ½ MS medium containing: sucrose 10 g L^{-1} (control) and 60 g L^{-1} and SA 10 and 30 mg L^{-1} as elicitors. At 3 days of culture T_0 = 0h was determined, samples were also evaluated in 12, 24, 36 and 48h. The cultures were collected and lyophilized to undergo the process of total isoflavonoids extraction and to be analyzed as to the content of these molecules in their extract. The extraction and chemical analysis of isoflavones was performed according to the technique described in item 2.5(c). Likewise, the quantification of the isoflavone content of the elicited samples used the same protocol described in item 2.5(d).

2.7. Statistical analysis

All culture experiments were conducted in triplicate. The germination experiments were completely randomized, composed of different concentrations, each concentration composed by three plates, 5 replicates per treatment, considering each plate an experimental unit. Hairy root cultures were run in quadruplicates for each line and the replicates of each line were combined in two, prior to extraction and analysis of isoflavones. Elicitation with hairy roots, were performed in triplicates. Isoflavone concentrations were determined by means of the standard curves for each one of the isoflavones analyzed in the samples, as well as the standard deviation of the samples under study.

3. Results and discussion

3.1. Effect of elicitor in roots length and isoflavone content in T. pratense plantlets

As an initial process, we tested the influence of the elicitors on the root growth and the relation with the content of isoflavones. In this way, Fig. 2 *(A)*, for sucrose using agar as gelling agent, it was noticed that for the control, the use of phytagel is a better choice to get longer roots, when the concentration of 30 g L^{-1} was employed, there was an increase compared to the control, but no difference if it correlates with the use of phytagel, in the concentration of 60 g L^{-1} it is possible to see a slightly difference if compare agar and phytagel, with the better growth in agar and in the following

concentrations, there is a drop or inhibition of the root growth in the *T. pratense* seedlings probably related with the concentration of sugar in the medium.

In tissue culture, agar is routinely used as a gelling agent, however, it is known that depending on the sources from which it comes, it contains several contaminants that end up increasing the chances of errors, making the comparisons more difficult, in this way, the Phytagel^{TM}, a selected grade of gelrite, being a very clean gelling agent and with effective protocols for various applications was used (VERAMENDI *et al*., 1997). Although it is known that a gelling agent, theoretically, should be an inert constituent of the culture medium (ARREGUI; VERAMENDI; MINGO-CASTEL, 2003), in relation to the root length in red clover seedlings it was possible to visualize that combined with higher concentrations of sucrose, media containing agar may even be more effective and an additional agent in the induction of root growth in these plants.

In the evaluation related to the isoflavone amount in red clover seedlings, it can be perceived, Fig. 3 *(A)*, that the addition of sucrose to the medium containing agar, promotes an intensification of the total isoflavones, obtaining the highest results in the concentration of 120 g L^{-1}, instead, when using phytagel, the peak of these molecules was in the concentration of 90 g L^{-1} of sucrose.

Sucrose is a carbon source and, is one of the five classes of essential substances needed for growth and development in plants, possessive also important signaling functions throughout all stages of the plant's life cycle, gene expression and enzymatic activities (SMEEKENS, 2000; SUJATHA; KUMARI, 2012). Nutritional deficiencies have a very pronounced effect on phenolic compound levels. The use of sucrose in high concentrations causes osmotic stress, reducing turgor and causing reactive oxygen species induction, hindering the availability of nutrients to the plant, and in plants like grape (*Vitis vinifera)* it can regulate the production of anthocyanin (RAMAKRISHNA; RAVISHANKAR, 2011).

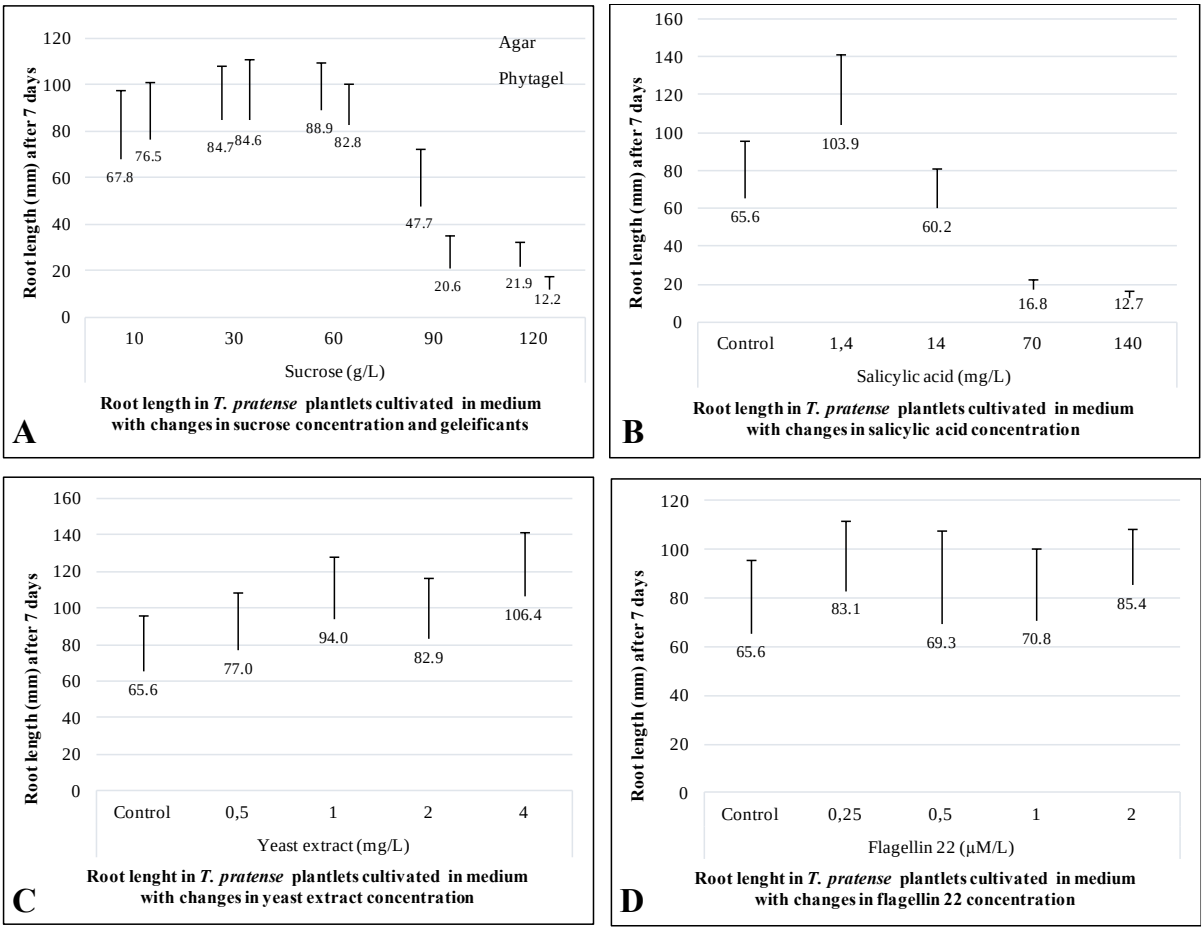

Figure 2. Root growth (mm) after seven days of cultivation in MS medium containing different concentrations of (A) Sucrose + agar or phytagel, (B) Salicylic acid, (C) Yeast extract and (D) Flagellin 22. Results are the mean of three replicates ± SD.

In the standard concentration of sucrose, at 10 g L^{-1}, Fig. 3 *(A)*, there is a small difference in the content of flavonoids, in the media containing the different gelling agents, with a minor increase in productivity when phytagel is used. Once sucrose 30 and 60 g L^{-1} was used, the preference would be the use of agar, presenting a stable concentration of isoflavones in both carbon sources quantities. This result modifies using 90 g L^{-1} sucrose, with a decay of the isoflavone in the medium with agar and increased concentration using phytagel. For 120 g L^{-1}, results similar to those of the control can be visualized when phytagel was employ and a peak in the isoflavonoids with agar.

Although the highest concentrations of sucrose + agar were the most effective, they were extremely damaging to the seedlings, which stopped growth at this concentration, as can be seen by the small size of the roots in Fig. 2 *(a)*. The seedlings developed in medium with phytagel showed a change in the color from the concentration

of 90 g L^{-1}, Fig.4 *(B)*, followed by the coloration of the extracts obtained from them Fig.4 *(C)*.

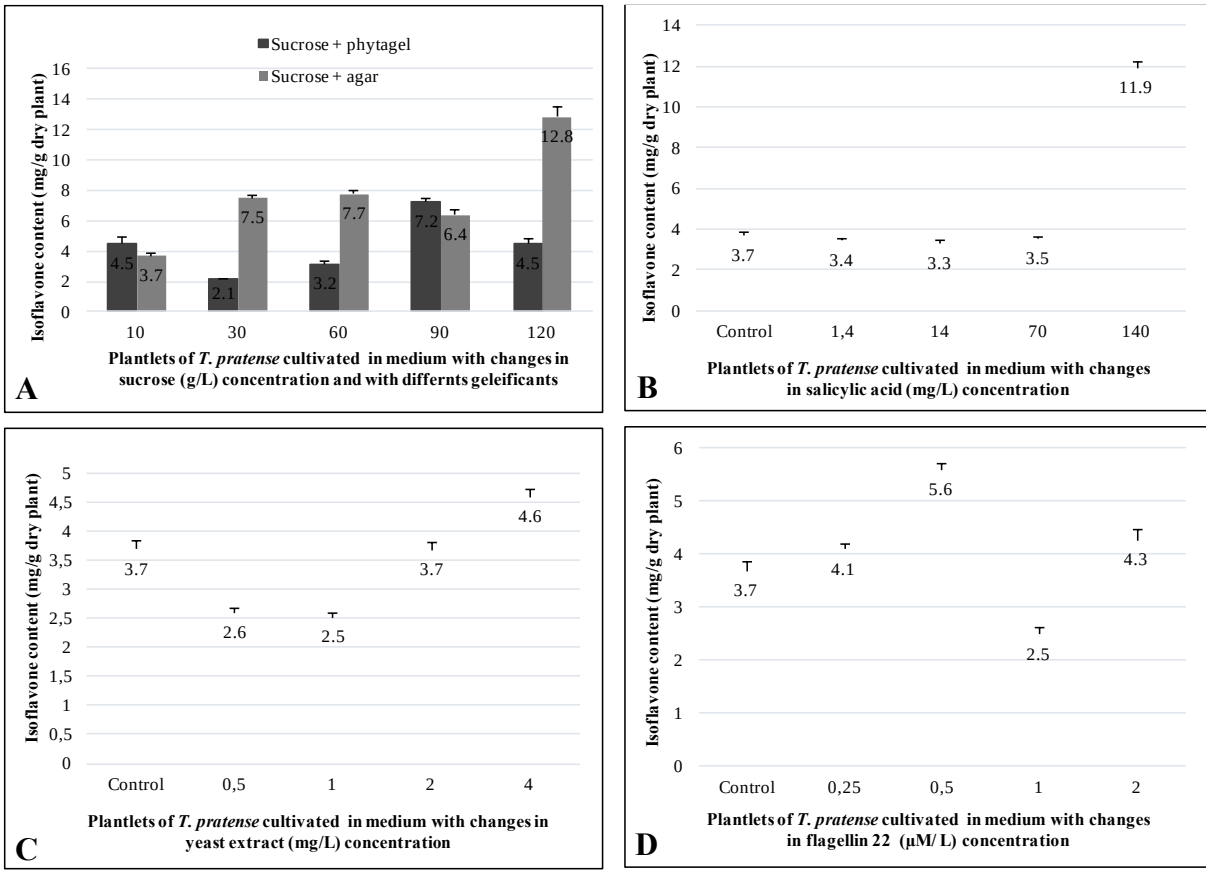

Figure 3. Effect of root length (mm) on *T. pratense* seedlings being cultivated in medium with the use of elicitors in different concentrations during the first seven days after germination. (A) Sucrose + phytagel or agar, (B) Salicylic acid, (C) Yeast extract and (D) Flagellin 22. Results are the mean of three replicates ± SD.

Sugars are signaling molecules, studies with the genome transcript profiling of *Arabidopsis thaliana* showed that the flavonoid and anthocyanin biosynthetic pathways are strongly up-regulated with sucrose (SOLFANELLI *et al*., 2006). This report explains the perceived coloration in plants and extracts (Fig. 4) of red clover seedlings and how sucrose can modulate the biosynthesis of anthocyanin.

SA is a plant growth regulator which has generated a range of physiological and metabolic responses in plants, modifying their growth, developmental status and inducing pathogens resistance (DURANGO *et al*., 2013; HAYAT *et al*., 2010), as can be seen in Fig. 2 *(B)*, at low concentration as 1.4 mg mL^{-1} ends up increasing the root growth, but at a concentration of 14 mg mL^{-1} the length is equal to the control, not promoting changes related with root length. At subsequent concentrations, the inhibition of growth is visualized as being more toxic to the roots.

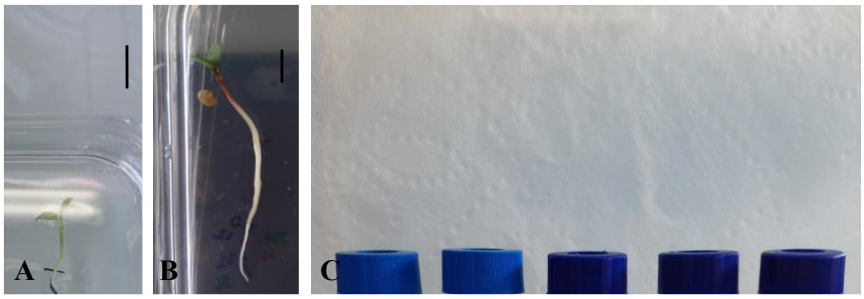

Figure 4. Color modification in the plantlets and the extracts from Sucrose plus phytagel as gelling agent in plantlets of *T. pratense*. (A) Plantlet cultivated in Phytagel + Sucrose 10 g L^{-1}, (B) Plantlet cultivated in Phytagel + Sucrose 90 g L^{-1}, (C) Flavonoid Extraction from the samples cultivated in Phytagel + Sucrose (left to right in crescent order of concentration, from 10 to 120 g $L^{-1}$of sucrose) Scale bar = 1 cm.

With regard to the isoflavone concentration when was used salicylic acid, Fig. 3 *(B)*, until de concentration of 140 mg L^{-1} of SA the yields of isoflavones were similar to control, however, using 140 mg L^{-1}, the amount almost tripled. Similar results were discovered with *Psoralea corylifolia* cell cultures, with similar concentration, doubling daidzein content (SHINDE; MALPATHAK; FULZELE, 2009a), for *Phaseolus vulgaris* cotyledons, the use of SA higher the phytoalexins from 50 to 100 times, since SA can promote the induction of defense compounds (DURANGO *et al*., 2013).

Yeast extract (*Saccharomyces cerevisiae*) is a water soluble portion of autolyzed yeast with intact B-complex vitamins and can be used as elicitors for plant cell or hairy root cultures (RAMIREZ-ESTRADA *et al*., 2016). For yeast extract, Fig. 2 *(C)*, and flagellin 22, Fig. 2 *(D)*, it is not possible to visualize morphological changes of the roots in the concentrations used, these fluctuations and small modifications could not be conclusive if there is or not the increase of the length with the increase of the concentration for YE and flagellin 22.

Using YE, Fig. 3 *(C)*, the intensification in the concentration was proportional to higher levels on total isoflavones, obtaining better results when using YE 4 mg L^{-1}, differing from control. However, the use of 2 mg L^{-1} presented the content of isoflavones equal to those of the standard and the other concentration, 0.5 and 1 mg L^{-1} obtained low isoflavonoid yield. In studies with *P. candollei* HR cultures, YE was the most efficient elicitor to exacerbate the concentrations of isoflavonoids, at 0.5 mg mL^{-1} after 3 days of culture (UDOMSUK *et al*., 2011).

Flagelin 22 (Flg22) is a 22-amino acid-long N-terminal part (QRLSTGSRINSAKDDAAGLQIA) of the principal protein of the bacterial flagellum,

and acts as a potent biotic elicitor in plants. As a bacterial microbe-associated molecular patterns activates FLS2-dependent signaling, triggering the same responses as the native protein from *Pseudomonas syringae* in plants like *Arabidopsis* (DE CONINCK *et al*., 2015; MAFFEI; ARIMURA; MITHÖFER, 2012).

For *T. pratense* seedlings, Fig. 3 *(D)*, using Flg 22 0.5 μM L^{-1}, it was obtained higher biosynthesis of isoflavonoids, and for 1 μM L^{-1} the results were worse. The other concentrations had analogous results when compared to the control. Nonetheless, due to the presence of these inconsistencies, more studies are necessary with the use of this substance.

3.2. Hairy root culture

Hairy roots (HR) are induced by transfer of T-DNA from the plasmid of *A. rhizogenes* (PETIT *et al*., 1983) to host tissue, resulting in root formation by virtue of auxin synthesis genes coded by bacterial DNA. The Ri plasmid of *A. rhizogenes* also elicits the synthesis of opines. The transformed nature of the roots can be checked by the high growth rate even without the use of hormones. Many times, they do not need incubation under light, have negative gravitropism and are fairly well stable in metabolite yield due to their genetic stability (FLORES, 1999).

A. rhizogenes strain A4TC24 the infected of *T. pratense* seedlings five days after germination, Fig. 5 *(A)*, the development of the adventitious roots was induced, 10 days after inoculation, with root formation at the site of infection. In Fig. 5 *(B)*, in more than 90% of the seedlings inoculated, it was possible to cut the adventitious roots grown at the infection sites and transfer them to solid medium, where they showed rapid growth and loss of the response to gravity (negative geotropism), Fig. 5 *(C)*. This rapid elongation is a phenotypic characteristic of the transformed roots, and rapid lateral branching is responsible for the increase in mass and accumulation in this roots (ESKANDARI-SAMET *et al*., 2012). Following with the protocol, the cultures were shifted to liquid culture medium, Fig. 5 *(D)*, where the multiplication rate was exponential, Fig. 5 (D to H).

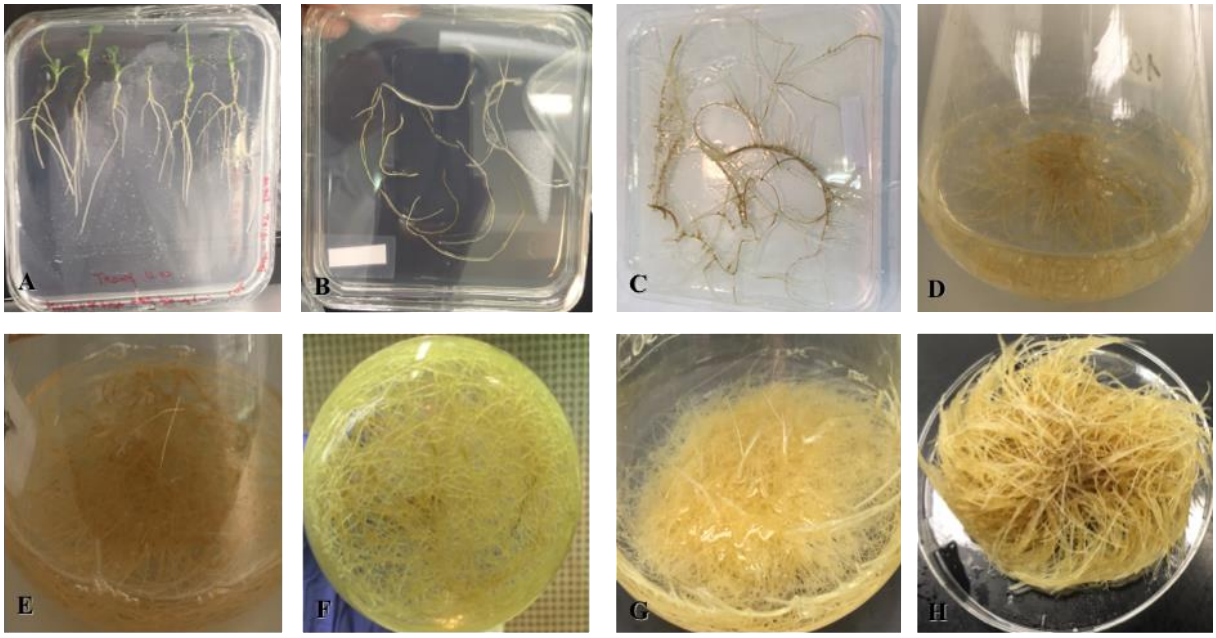

Figure 5. Adventitious root induction and establishment of hairy root of red clover induced by *A. rhizogenes* infection. A) Induction in the seedlings, 10 days after infection. B) Roots transferred to the medium without growth regulators, 30 days after the induction. C) The development of the roots after 1 week in medium. D) Transference to liquid medium, 50 days after infection. E and F) Hairy root after one week in liquid medium (above and below of the bottle). G and H) The hairy root cultures 3 weeks in liquid medium.

a) Isoflavone identification

Thirteen cultures were harvested after the second month of cultivation to proceed the verification of productivity and total isoflavonoids content by HPLC-ESI-MS/MS. According to analyzes, were produced by this cultures 16 compounds in chromatogram (Table 1). It is possible to identify the presence of aglycones and conjugated forms such as glycosides and glucosides malonates, confirming the information previously published in identifications carried out in roots of the same species (SAVIRANTA *et al.*, 2010).

Table 1. Isoflavonoids identified in *T. pratense* hairy root cultures lineages by HPLC-ESI-MS/MS.

N° compound	**t_R[a]**	**Aglycone MH+[b]**	**(*m/z*)[c]**	**Product ions[d]**	**Identification[e]**
1	9.3	315	563	248	3',7-Di-O-Methylorobol (5Hydroxy-3-(4-hydroxy-3-methoxyphenyl)-7-methoxy-4H-chromen-4-one) Malonyl Hexose
2	9.75	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose
3	11.4	269	431	162	Formononetin - hexose
4	11.66	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose
5	12.05	283	531	248	Pseudobaptigenin- Malonyl Hexose
6	12.33	285	285	-	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin

7	12.41	269	517	248	Formononetin -Malonyl Hexose
8	13.26	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose
9	13.52	299	547	162	Irilone/Afrormosine-Malonyl Hexose
10	13.54	301	549	248	Pratensein/ 3 O Methylorobol (5,7-dihydroxy-3-(4-hydroxy-3-methoxyphenyl)-4Hchromen-4one) Malonyl Hexose
11	14.41	285	533	248	Biochanin/calycosin/maackiain/Glycitein/Prunetin/Texasin - Malonyl Hexose
12	14.58	301	301	-	Pratensein/ 3 O Methylorobol (5,7-dihydroxy-3-(4-hydroxy-3-methoxyphenyl)-4Hchromen-4one)
13	15.51	283	283	-	Pseudobaptigenin
14	**15.92**	**269**	**269**	**-**	**Formononetin**
15	**18.51**	**285**	**285**		**Biochanin A**
16	19.66	417	417	-	Daidzein

[a] rT: retention time in minutes

[b] Aglycone MH+: molecular ion weight provided by ESI-MS in a positive mode after the lost of the sugar molecule.

[c] m/z: [M+H]+: molecular ion weight provided by ESI-MS in a positive mode.

[d] Product ions: fragment obtained by MS/MS with the collision energy.

[e] Identification: suggested structure of the compound based on rT and the m/z confirmed with a standard (bold) or from MS and MS/MS spectra deduction.

As can be seen in Fig. 6, all the cultures were isoflavone producers, however, the compounds with higher production were the isomers of biochanin (**4**), presenting percentages of 26.3 to 58 % of the total isoflavonoid content detected in these strains. The second molecule with the greatest increase was formononetin aglycone (**14**), from 10.1 to 36.5 %, being accompanied by other isoflavonoids, such as Irilone/ afrormosine-malonyl hexose (**9**), 4.4 to 17.7 % and biochanin isomers (**6**), 6.7 to 13.5% of the total isoflavonoid content. The other molecules were produced in minor quantities and some of the cultures were unique producers of certain compounds, as was the case of HR10, the only one to produce compound **12** and **3**, and HR5, unique to biosynthesizing the compound **16**.

The differences between the cultures were morphological and in the growth capacity, but mainly, biochemical, demonstrating high variability in the content of isoflavonoids. The dissimilarities can be due to two factors, firstly, seeds were used to start the crops, and each lineage was the result of the infection of one plant. As it is known, herbaceous plants possess heterogeneous populations with genetic

differentiation and for Fabaceae family, higher genetic diversity levels were detected (HAMRICK; GODT, 1996; KONGKIATNGAM *et al.*, 1995).

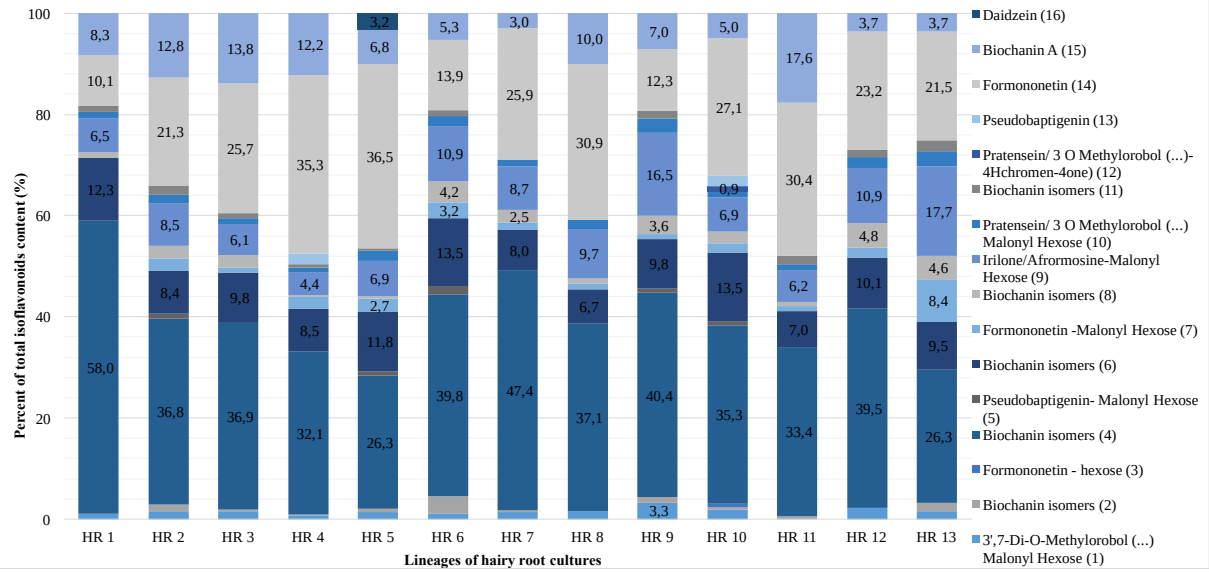

Figure 6. Graphic representation of isoflavonoids profile of *T. pratense* hairy roots lineages showing the percentage of the contents. Values less than 3 % have been removed from the view.

Another important point is related to the effect of *A. rhizogenes rol* B gene that presents varying levels of expression between the different rows of roots and thus alternates the internal proportion of auxins and cytokinins or even the sensitivity to this hormonal balance in each transformed genotype (FU *et al.*, 2005), also, because of the uncertainty of T-DNA integration into the host plant genome, the HR derived normally show different accumulation patterns of secondary metabolites, variation in growth rate and productivity between the clones. Studies also showed that, even if the cultures comes from a single root, they will present a certain heterogeneity (HU; DU, 2006).

b) Isoflavone content in first five months of culture

In order to uncover the red clover root cultures in the first months after the transformation, four samples were selected, observing the differences reported in the previous analyzes (Fig. 6), that presented a good multiplication rate and stability. At the time of subculture, each four weeks, the cultures were harvested and stored for later determination of the isoflavonoids production at the end of the fifth month in liquid culture medium.

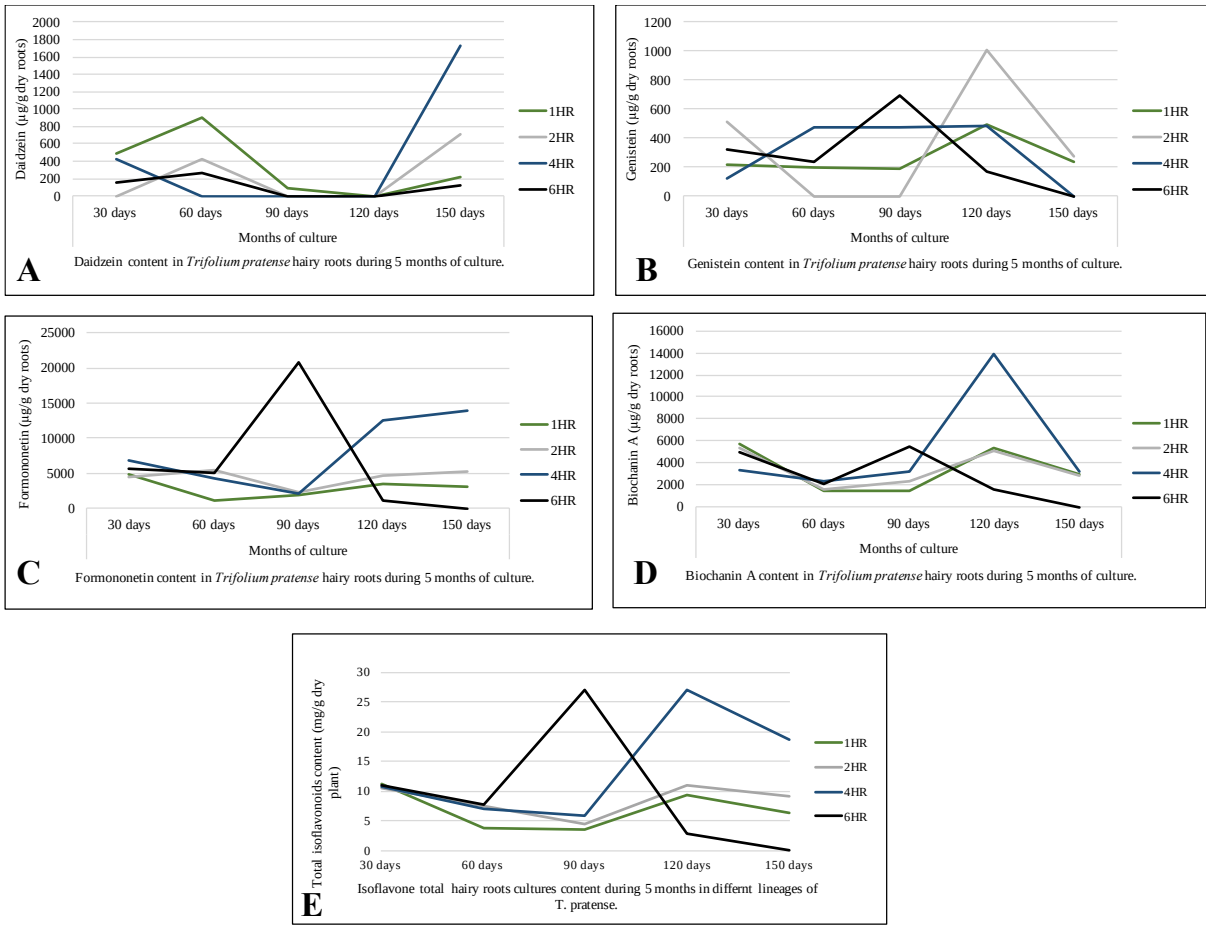

Figure 7. Isoflavones in *T. pratense* HR lineages during the first 5 months of cultivation. (a) Daidzein; (b) Genistein; (c) Formononetin and (d) Biochanin A content.

In this way, the 1H, 2HR, 4HR and 6HR lines were analyzed for five months, Fig. 7 (*A* to *D*), and the amount of daidzein, genistein, formononetin and biochanin, their fluctuations and differences over the months, also, the total content of these four molecules, Fig. 7 (*D*), to demonstrate these lineage-by-lineage discrepancies over the months.

The main isoflavones in red clover are biochanin A and formononetin, and the second is more abundantly in roots (SAVIRANTA *et al.*, 2008). This affirmation is modified when related with transformed roots, according to the analyzes, each of the cultures presented a metabolite as a major, 4HR for daidzein at the end of the 150 days, Fig. 7 (*A*), 2HR for genistein Fig. 7 (*B*) at 120 days of culture, 6HR presented as a major constituent formononetin after 3 months of culture, Fig. 7 (*C*), and biochanin A was best produced by 4HR cultures at 4 months in liquid medium, Fig. 7 (*D*).

The total content of these four isoflavones was verified, Fig. 7 (*D*), and the month of greatest productivity was the third, due to the high production of formononetin in 6HR, followed by the 120 days, by the 4HR sample, the other lineages show small amounts of this molecules.

Studies of isoflavones over the months have not been found, however, reports have shown that these strains can maintain genotypes and stability for extended periods, only losing this ability with drastic changes of the media or the addition of plant hormones that will induce metabolic modifications and to cause a disorganization in the cultures, which occurred with *Datura* and *Nicotiana* cultures (DORAN, 1997).

c) Extraction and chemical analysis of isoflavones

After analyzing the samples for five months, in order to make the characterization lineages profile and biosynthesis, two of them were determined for tests with elicitors, after the specific days of elicitation they were harvested, stored and extracted for the evaluation in daidzein, genistein, formononetin, biochanin A and the total content of this four molecules during the days in treatment, using the UPLC.

With the exception of the 4HR culture when using salicylic acid 10 mg L^{-1}, Fig. 8 *(E)*, all the others presented a curve of decay of the total content. The highest verifications were generated by the use of sucrose 60 g L^{-1} in 4 HR, Fig. 8 *(C)*, and subsequently, 8HR, Fig. 8 *(D)*, (27.883 and 21.209 mg g^{-1} dry material, respectively). Regarding the standard, the use of salicylic acid 30 mg L^{-1} in 4H lines, Fig. 8 *(G)*, were the less productivity inducer of these molecules, with the highest content in 5.237 mg g^{-1} dry roots.

Sucrose is the most widely used carbon source in the world due to its effective absorption and hydrolysis of glucose and fructose by the plant cells for assimilation, being able to act as carbon sources or as osmotic regulators, and may cause tissue toxicity, depending on the dosage (PETROVA *et al*., 2015). This way, using sucrose 10 g L^{-1}, control of 4HR line, fig. 8 *(A)*, in a general view, showed the isoflavone concentration decreased during the course of root growth, ranging from 11.209 at 0h, up to 2.529 mg g^{-1} of dry mass, the higher isoflavone detected was daidzein, and as the concentration of this decreased, biochanin A was slowly increasing.

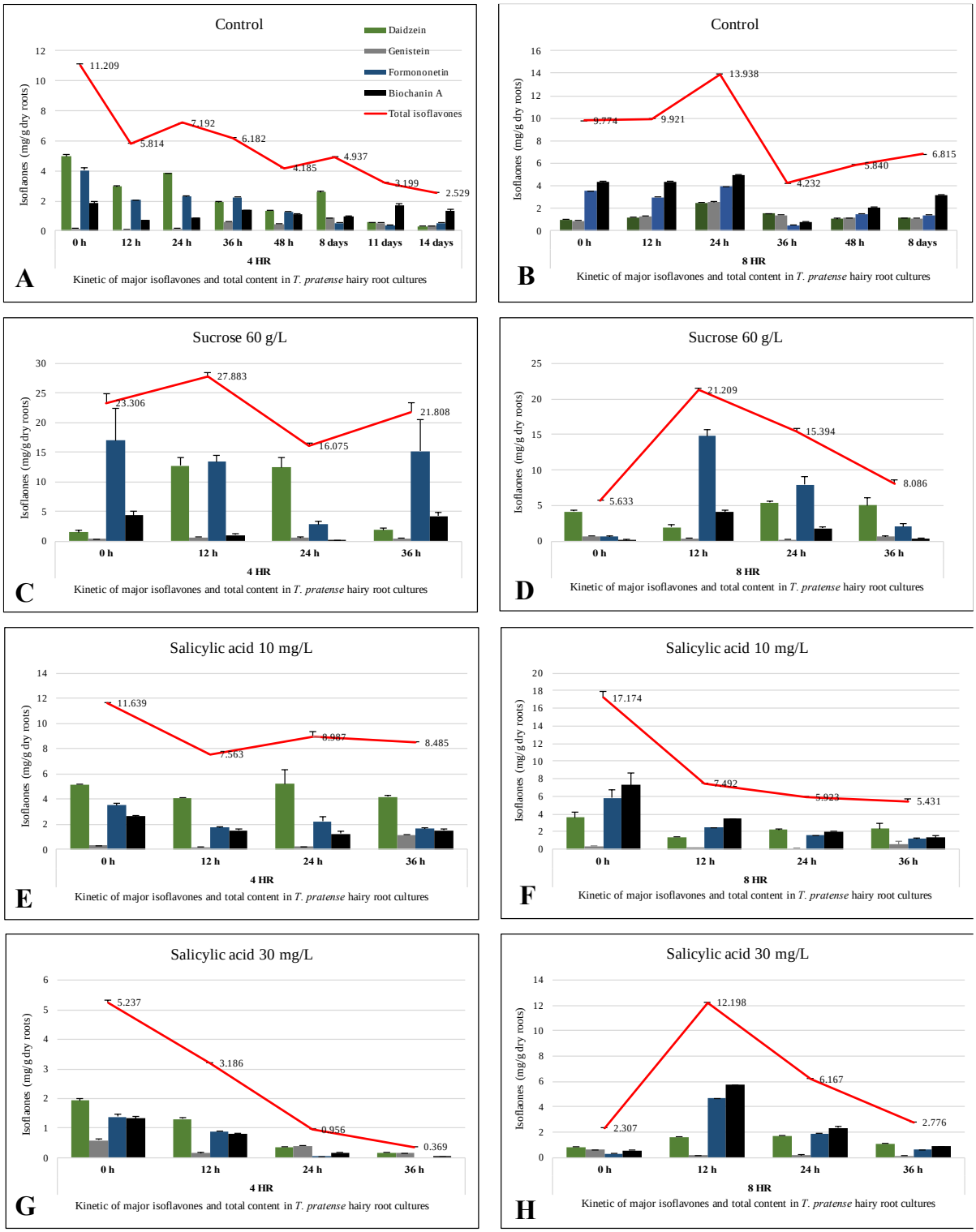

Figure 8. Effect of sucrose 10 (control) and 60 g L^{-1} and salicylic acid 10 and 30 mg L^{-1} on the accumulation of major isoflavones and in the total content. Results are the mean of three replicates ± SD.

For the 8HR lines, Fig. 8 *(B*), the control started with a total concentration of isoflavones of 9.774 mg g^{-1} and at 24 h in culture had an intensification, caused by the

augmentation of all isoflavones, then reducing and growing the concentration slowly until reaching 6.815 mg g^{-1} at 8 days of treatment, with a rise of biochanin mainly, being that the major molecule for this culture in the control is biochanin A. *Psoralea corylifolia* HR clones cultured with high levels of sucrose could increase the yield of daidzein, although, this caused the decrease of genistein levels when used in the presence of PO_4^{-3} at low concentrations (SHINDE; MALPATHAK; FULZELE, 2010), analogous to our results, in which genistein levels were near zero.

In comparison, the use of sucrose 60 g L^{-1} in 4HR caused an exacerbation of productivity at 12 hours of treatment, reaching 2.5 fold the highest concentration found in control. Different results were discovered in *Pueraria phaseoloides* transformed roots, were sucrose 3% concentration was the best in the accumulation of isoflavones after 16 days of treatment (LIANG; SHI; QI, 2004). In red clover, the profile of the phytohormones was also modified, increasing the production of formononetin in the standard in 4 times, genistein almost non-existent, and generally, modifying all isoflavones configuration in 4HR.

For 8HR, this concentration of sucrose was also with the highest peak of total isoflavones content and, like the other lineage evaluated, modified its appearance, making the formononetin majority, without production of genistein and with biochanin A appearing only at 12 and 24 hours of elicitation. *Hypericum perforatum* L. root cultures studies demonstrate that high concentrations of sucrose can stimulate the production of total phenols and flavonoids in the cultures (CUI *et al*., 2010).

In recent years, SA it has been used in HR of *Cichorium intybus* (sesquiterpene lactones and sonchuside A) (MALARZ; STOJAKOWSKA; KISIEL, 2007); *Azadirachta indica* (azadirachtin) (SATDIVE; FULZELE; EAPEN, 2007); *Psoralea corylifolia* (daidzein and genistein) (SHINDE; MALPATHAK; FULZELE, 2009b) and *P. candollei* (isoflavonoid) (UDOMSUK *et al*., 2011) to update on enhancement of secondary metabolites in the cultures.

In red clover HR cultures, the use of SA 10 mg L^{-1} in 4HR reached the same levels of control, however, instead of decay, it maintained the final concentration in 8.482 mg g^{-1} dry roots, and the profile remained the same, with daidzein as the major

and levels similar to those of the control as well. For 8HR, the configuration was also preserved, with the exception of genistein that presented very low yields, the peak induced by salicylic acid in this dosage was 17.174 mg g^{-1} dry mass, above the control, but after that, the concentration was decreasing.

HR evaluations of *P. corylifolia* (SHINDE; MALPATHAK; FULZELE, 2009b) showed that the elevation in the concentration of SA causes a reduction in the levels of daidzein and genistein, agreeing with this information, as previously mentioned, the use of SA 30 mg L^{-1} in 4HR had the lowest accumulation of the isoflavone content, with a drop in concentration to almost zero. However, for 8HR, it started with a low accumulation, but at 12h it had a peak, with a concentration somewhat lower than the control, and then the decrease occurred until returning to the initial values, also, the profile remained similar to control, with higher production of biochanin A, but no production of genistein.

Studies with HR of *P. corylifolia* (UDOMSUK *et al.*, 2011), showed that the elicitation for these cultures was independent of the concentration or duration of the treatment used. Our research demonstrates that *T. pratense* HR diverged from this assertion, demonstrating that elicitation with SA is dependent on lineage, dosage and on elicitation time to generate a different response, as shown in Fig. 8 (*E -H*).

Salicylic acid, an important plant signaling compound involved in plant resistance in response to various pathogenic attacks (HAYAT *et al.*, 2010), however, according to our findings, stimulatory effect of this molecule is determined for each plant, having specific values and times so that overproduction occurs; otherwise, there will be depletion of the secondary metabolites responsible for this defense.

4. Supplementary material

4.1. Media

All media were calculated for a volume of 1L and were sterilized by autoclaving for 20 min at 120 °C. Antibiotics, hormones or other components, were added after autoclaving the media.

a) Media for plant culture

MS medium (MURASHIGE; SKOOG, 1962):

4.49 g Murashige and Skoog medium basal salt mixture including vitamins and microelements (Duchefa, Haarlem, The Netherlands), 30 g sucrose pH 5.8, 1% agar (Difco, Detroit, USA) for solid medium. Sterilized by autoclaving.

b) Media for bacterial culture

LB medium:

5 g Yeast extract (Difco), 10g Tryptone (Difco), 10 g NaCl, 10 g agar (Difco) for solid medium, pH 7.0. Sterilized by autoclaving.

YEB medium:

5 g Beef extract (Difco), 1 g Yeast extract (Difco), 5 g Peptone (Difco), 5 g Sucrose, 2mM $MgSO_4$, pH 7.2. Sterilized by autoclaving.

5. Conclusion

This work presents the report of plantlets and *A. rhizogenes*-mediated transformation of *T. pratense* related with isoflavone content, both total or the main aglycone isoflavones present in seedlings and hairy root crops, with and without the presence of elicitors. Seedlings, had better root growth in the presence of salicylic acid 1.4 mg L^{-1} and yeast extract 4 mg L^{-1}, however, the highest accumulation of total isoflavones was found using sucrose 120 g L^{-1} and SA 140 mg L^{-1}. The hairy root cultures showed fast growth rates and exhibited different biochemical profiles isoflavonoids and, when cultivated for five months, these profiles changed in the presence of elicitors, demonstrating high-level production of total content when was sucrose was added at 60 g L^{-1} in the two lineages analyzed.

Thus, we conclude that the use of *A. rhizogenes* strain A4TC24 with the use of sucrose 60 g L^{-1} could be used to produce isoflavonoids in *T. pratense* transformed roots and yeast, as an initial process, serving as the starting point for large-scale cultures, in bioreactors or the transformation with genes of intermost for the overproduction of these molecules.

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Os experimentos conduzidos com *R. bifida* foram desempenhados em laboratórios na França (LFR) e no Brasil (LBR) e variaram quanto à concentração de montanina. As plantas de *Rhodophiala bifida* foram coletadas no Brasil nos anos de 2013, 2014 e 2015, destas, as amostras de 2014 foram introduzidas no cultivo *in vitro* por meio de regeneração (Brasil) e, posteriormente, foram desenvolvidos os trabalhos *in vitro*, químico e molecular durante o Doutorado Sanduíche com as amostras da mesma coleta.

Para uma melhor visualização dos resultados como um todo, foram utilizados os maiores incrementos de montanina detectados nos bulbos selvagens (amostras de 2013, 2014 e 2015 coletados no Brasil comparadas com as cultivadas em casa de vegetação na França), cultivadas *in vitro* (regeneradas no Brasil comparadas com as germinadas *in vitro* na França) e por fim, aclimatizadas (após a regeneração em ambos os países) que foram apresentados na Fig. 3.

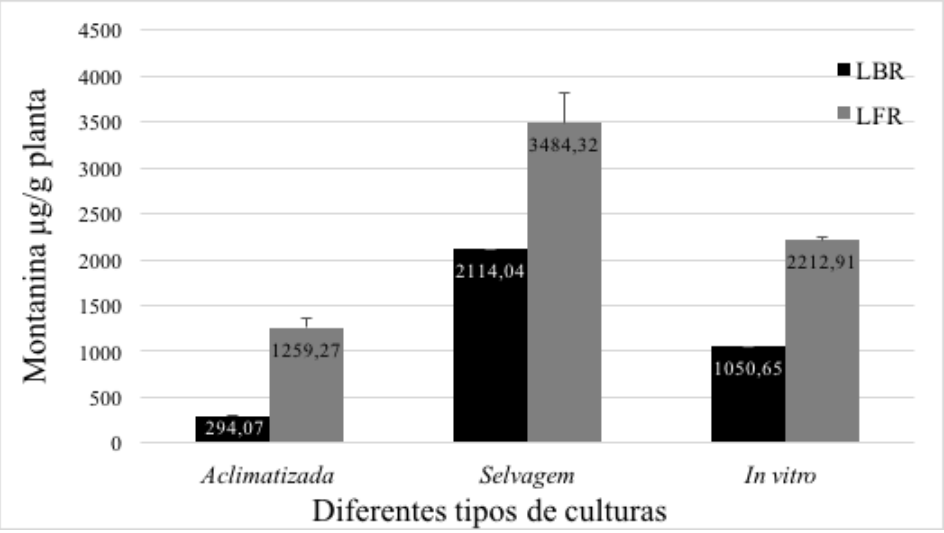

Figura 3. Análises de quantificação da montanina presentes em bulbos de *R. bifida* cultivados em diferentes tipos de cultivos.

Plantas selvagens apresentaram conteúdo de montanina inferior aos das plantas selvagens cultivadas em casa de vegetação por 9 meses, mesmo sendo pertencentes à mesma população, os diferenciais entre elas foram solução nutritiva, umidade, irrigação e temperaturas controladas fornecidas às plantas da estufa durante o período de desenvolvimento da planta. Um estudo com *Leucojum aestivum* evidenciou que modificações na temperatura de cultivo duplicaram o percentual de galantamina

produzido pela planta, já para a licorina, ocorreu uma redução de 2,2 x (GEORGIEV *et al*., 2012), mostrando que a temperatura durante o crescimento do vegetal, tem efeito significativo na biossíntese destes alcaloides.

A disponibilidade de nutrientes é um dos principais fatores envolvidos no cultivo de plantas, tanto *in vitro*, quanto *ex vitro* e afeta diretamente a acumulação de biomassa, assim como a biossíntese de metabólitos (PAEK; CHAKRABARTY; HAHN, 2005). As soluções nutritivas contêm os macro e micronutrientes essenciais para o crescimento dos vegetais, possibilitando que as peculiaridades fisiológicas relacionadas à biossíntese dos alcaloides sejam sanadas, como o que pôde ser visualizado com o cultivo *in vitro* de *L. aestivum* (PAVLOV *et al*., 2007).

As sementes utilizadas no estudo, da variedade Spathacea, quando germinadas e avaliadas, exibiram o dobro do conteúdo de montanina observado nas plantas regeneradas cultivadas *in vitro*. Estes resultados podem ser devido a diferenças na variedade das plantas, meio de cultivo ou estádios de desenvolvimento totalmente diferenciados.

Em uma pesquisa com *Narcissus confusus*, foi relacionado o estádio de diferenciação da planta ao seu conteúdo de alcaloides, demonstrando que plantas regeneradas a partir de culturas de brotos, conforme o processo de regeneração realizado com a *R. bifida*, apresentaram maiores teores dos alcaloides analisados (galantamina, haemantamina, *N*-formilgalantamina e tazetina) (SELLÉS *et al*., 1999). Relativo a diferentes variedades, comprovou-se que plantas de *L. aestivum* de populações selvagens apresentaram uma variabilidade na concentração do alcaloide majoritário (galantamina) em cerca de 75 vezes (GEORGIEVA *et al*., 2007).

De acordo com o que foi considerado quanto ao montante presente em plantas que passaram por regeneração *in vitro* e posteriormente foram transferidas para casa de vegetação, o conteúdo de montanina é relativamente baixo. Contrastando com estes resultados, as plantas *Scopolia parviflora*, produtoras de alcaloides tropânicos exibiram concentrações relativamente altas destes alcaloides nos rizomas de plantas aclimatizadas (KANG *et al*., 2004).

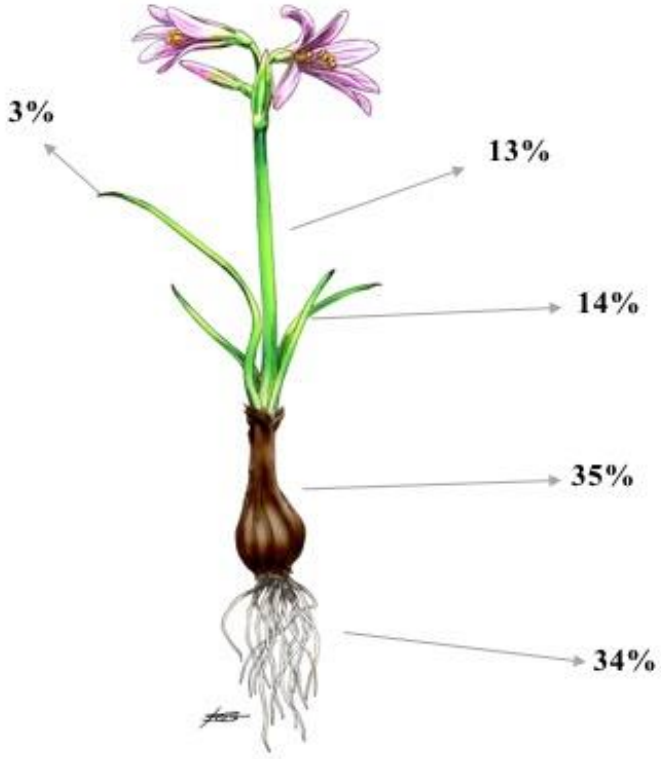

Figura 4. Conteúdo total de montanina presente em *R. bifida*, de acordo com os percentuais identificados em cada um dos órgãos analisados. Ilustração: Domênico Gay.

Sabe-se também que as maiores concentrações de montanina dos tecidos foram localizadas nas raízes e bulbos, *Fig. 4*, dessa forma a extração acaba por consumir a planta inteira para que seja concretizada. Com *Crinum macowanii*, os resultados foram semelhantes, demonstrando que 75% dos alcaloides encontrados nesta Amaryllidaceae, estavam em maiores concentrações nos bulbos e em segundo, nas raízes. (ELGORASHI; DREWES; VAN STADEN, 2002), porém, divergiram dos estudos em *Sternbergia lutea*, nos quais, as maiores determinações de licorina ficavam nas folhas (AMICO; STEFANIZZI, 1978).

Dessa forma, dentre as alternativas estudadas, a melhor forma de cultivo visando alta produtividade para o alcaloide montanina seria a coleta das plantas selvagens e cultivo em casa de vegetação ou estufas climatizadas, com solo, umidade, irrigação e nutrição controlados. Avaliações usando bulbos de *Narcissus* observaram que a aplicação de fertilizantes com níveis padronizados de nitrogênio e potássio causaram um aumento significativo dos níveis de galantamina quando comparados ao controle (LUBBE; VERPOORTE; CHOI, 2012).

O processo *in vitro* apresenta grande potencial, como pôde ser visualizado nos estudos relatados no Capítulo I, porém, no caso da *R. bifida*, ainda deverão ser exploradas outras alternativas relativas a esse método de produção de biomassa e montanina.

Considerando em seu conjunto, os dados relativos às plantas de *R. bifida* conectados aos estudos do nosso grupo, relativos às atividades biológicas dos alcaloides de Amaryllidaceae possibilitam que se tenha conhecimento acerca da melhor forma de cultivo intencionando grande produtividade por parte destas plantas. A identificação das moléculas presentes, assim como dos genes da rota nas plantas de *R. bifida* possibilitam que os próximos estudos referentes a estas enzimas possam ocorrer e dessa forma uma melhor elucidação de toda a biossíntese dos alcaloides do tipo montanina.

Avaliando os experimentos realizados com as plantas de *T. pratense* e fazendo uso dos resultados que se destacaram em cada um dos estudos, buscou-se analisar o conteúdo total de isoflavonas (daidzeína, genisteína, formononetina e biochanin A) para evidenciar a melhor escolha de cultivo para esta espécie objetivando a produção destas moléculas.

As culturas celulares e de raízes transgênicas são fontes alternativas bastante promissoras para a produção de metabólitos secundários de alto valor e importância industrial (RAO *et al*., 2002). Dessa forma, pôde-se visualizar na *Fig. 4* que as culturas de raízes apresentaram os maiores incrementos do conteúdo total de isoflavonas, até mesmo correlacionados às plantas selvagens. Em uma pesquisa realizada com as raízes de plantas de trevo-vermelho (SAVIRANTA *et al*., 2010), relatou-se que a concentração de isoflavonas agliconas foi 39 vezes maior nas raízes de plantas crescidas em potes em comparação com as raízes de plantas do campo, sendo que o órgão de maior abundância das isoflavonas majoritárias é nas raízes (SAVIRANTA *et al*., 2010).

As culturas de raízes em cabeleira (*hairy roots*) são um sistema experimental bem estabelecido e, principalmente, têm fornecido muitas informações quanto ao metabolismo radicular e a sua regulação (FLORES, 1999). Concordando com nossos resultados, para espécies de *Glycyrrhiza*, as culturas de raízes são as mais produtivas fontes de novas isoflavonas (VEITCH, 2007) e em *Psoralea corylifolia*, o uso de

elicitores nas culturas de raízes promoveu um aumento significativo de daidzeína e genisteína frente ao controle (SHINDE; MALPATHAK; FULZELE, 2009a).

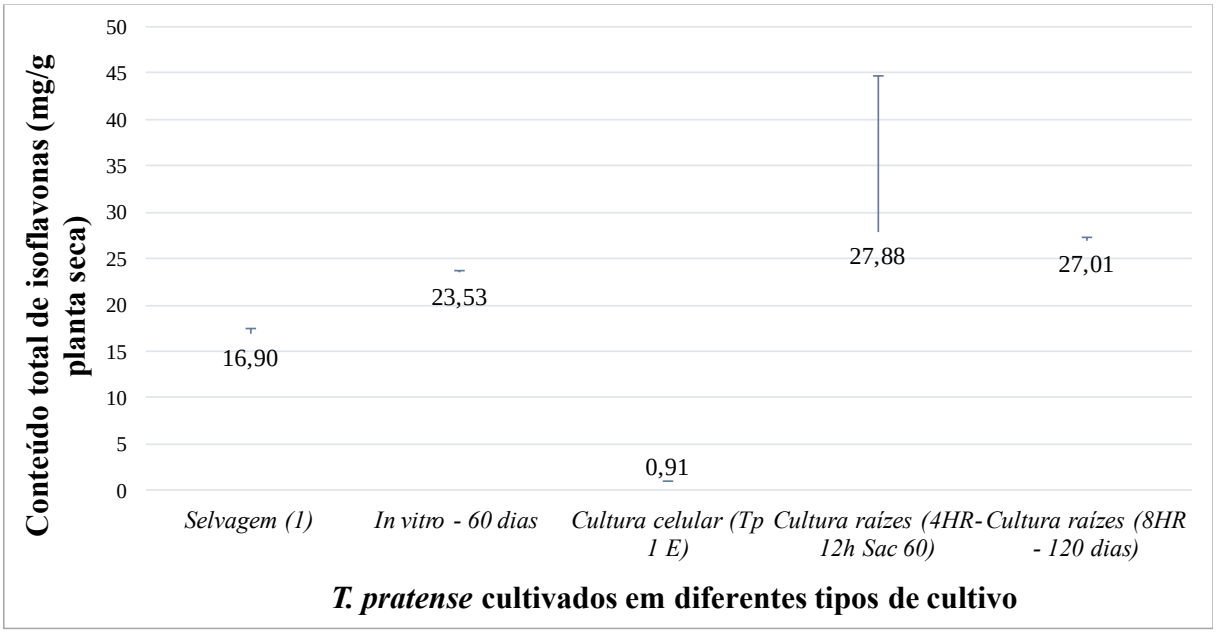

Figura 4. Avaliação do teor de isoflavonas presentes em *T. pratense* cultivados em diferentes tipos de cultivo.

Conforme é demonstrado na *Fig. 4*, as culturas radiculares das linhagens 4HR e 8HR exibiram resultados superiores aos demais tipos de cultivos em duas situações determinadas, após 12 horas de elicitação com sacarose 60 g L^{-1} e durante o cultivo de longo prazo, no qual as culturas foram analisadas mensalmente e exibiram pico de produtividade ao final de 120 dias de crescimento.

Várias pesquisas fizeram uso de elicitores visando o aumento da produção dos metabólitos secundários em cultura de tecidos, células ou órgãos, o que normalmente ocorre devido ao fato de que o crescimento geralmente sofre maior inibição pelo agente estressor do que a fotossíntese, alocando a fixação de carbono para o metabolismo secundário. Deficiências nutricionais têm um efeito bastante pronunciado quanto aos níveis de compostos fenólicos. A utilização de sacarose em altas concentrações causa estresse osmótico, dificultando a disponibilidade de nutrientes para a planta, por exemplo, em *Vitis vinifera* ela regula a produção de antocianina (RAMAKRISHNA; RAVISHANKAR, 2011).

De acordo com os resultados obtidos, também podemos observar (Fig. 4), que os valores encontrados para as plantas selvagens foram inferiores às plântulas cultivadas *in*

vitro, sem o uso de elicitores. Já em pesquisa realizada comparando o incremento de isoflavonas em calos e plântulas de *T. pratense*, determinou-se que maior conteúdo destas moléculas foi encontrado nos calos oriundos de explantes cotiledonares (GU; CHEN, 2006).

As culturas celulares que tiveram maiores teores de isoflavonoides contiveram resultados cerca de 30 vezes menores que as culturas de raízes avaliadas e quase 19 x inferiores aos obtidos nas plantas selvagens, um relato com calos e culturas celulares de *T. pratense* tetraploide, observou que em comparação com o cultivo em campo, o conteúdo de isoflavonoides foi muito inferior (ÇÖLGEÇEN *et al*., 2014).

Estudos com culturas celulares de *Pueraria lobata* apresentaram teores de isoflavonas totais inferiores no material crescido *in vitro* quando comparado com as raízes das plantas selvagens (LIU; LI; DEBERGH, 2002). Já em culturas celulares de *P. corylifolia* obteve-se um aumento do conteúdo de fitoestrógenos quando do uso de elicitores, possibilitando que para a daidzeína, chegasse a 5 vezes o valor encontrado no controle (SHINDE; MALPATHAK; FULZELE, 2009b).

Estes resultados provavelmente sejam devido ao contato célula-célula, envelhecimento e a diferenciação celular limitada que ocorre tanto em calos quanto em culturas celulares originadas deles, demonstrando que o grau de diferenciação e organização do tecido celular são relacionados positivamente com a acumulação destes fitoestrógenos (FLORES, 1992; LIU; LI; DEBERGH, 2002; PARR, 1988).

Sob o ponto de finalização deste trabalho, podemos avaliar os estudos do trevo-vermelho como elucidativos do ponto de vista de forma de cultivo visando produtividade de fitoestrógenos, com protocolos estabelecidos para germinação *in vitro,* culturas celulares e culturas de raízes, possibilitando que se saibam alternativas para a produção de moléculas sem maiores danos ao meio ambiente, com culturas limpas e de alto rendimento.

Por meio da elaboração, execução e análise das pesquisas durante estes quatro anos, podemos destacar:

Rhodophiala bifida:

- As plantas de *R. bifida* sofrem organogênese direta quando os explantes bulbares são cultivados em meio MS modificado com o uso de ANA (0,1 mg L^{-1}) e BAP (0,5 mg L^{-1}).
- Observou-se que após 90 dias em cultivo, após a regeneração, ocorre maior incremento do conteúdo de montanina quando cultivadas *in vitro*.
- Plantas selvagens coletadas no verão, com menor precipitação e umidade podem apresentar um aumento de montanina quando cultivadas no campo.
- Foram determinados os protocolos para germinação, regeneração, multiplicação e aclimatização das plantas, apresentando montanina em todas as etapas do processo de micropropagação e, posteriormente, no cultivo em casa de vegetação, mesmo que em concentrações baixas.
- O perfil de alcaloides presentes foi determinado em diferentes estruturas e revelou-se bastante diversificado quanto aos percentuais (TIC) apresentados por cada órgão, determinando grande variabilidade até mesmo no conteúdo de montanina, a molécula majoritária.
- Os genes *Rb*N4OMT e *Rb*N4OMT apresentaram similaridade com as sequências de proteínas dos genes identificados em *N.* aff. *pseudonarcissus*, os resultados de expressão do gene *Rb*N4OMT foram nos bulbos das plântulas e aclimatizados. Para *Rb*CYP96 o pico de expressão foi em raízes de plantas selvagens.
- A melhor forma de cultivo dentre aquelas avaliadas foi a coleta dos bulbos no verão e a transferência para casa de vegetação para serem cultivados com o uso de solução nutritiva por seis meses, para então serem coletados e extraídos.

Trifolium pratense

- Foram estabelecidos os protocolos para germinação, culturas celulares e culturas de raízes de trevo-vermelho.

- A maior formação de calos friáveis foi visualizada em meio MS suplementado com ANA 2,5 mg L^{-1} e cinetina 0,5 mg L^{-1}, a partir dos quais foram iniciadas as culturas celulares.
- O perfil químico das linhagens de culturas celulares apresentou grande variabilidade, principalmente quando comparados aos das plantas selvagens.
- As plantas selvagens coletadas em épocas de inverno, com tempo frio e chuvoso, apresentam maior biossíntese das isoflavonas avaliadas.
- O protocolo para transformação das raízes foi realizado com *Agrobacterium rhizogenes* strain A4TC24 e produziu linhagens com grande teor de isoflavonoides que se mantiveram estáveis nos primeiros 5 meses de cultivo.
- Foi possível determinar o perfil das isoflavonas presentes nas dez linhagens de culturas de raízes de *T. pratense*.
- Linhagens 4HR, após 12 horas de cultivo com sacarose 60 g L^{-1} como elicitor, aumentaram em 5 vezes o conteúdo do controle, determinando, juntamente com as culturas de raízes da linhagem 8HR, após 120 dias de cultivo, os melhores meios de cultivo relacionados à produção de isoflavonoides em trevo-vermelho deste estudo.

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