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Novas abordagens na micropropagação de *Arracacia xanthorrhiza* Bancr. ("mandioquinha-salsa")

Florianópolis
2023

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Dissertação submetida ao Programa de Pós-Graduação em Recursos Genéticos Vegetais da Universidade Federal de Santa Catarina para a obtenção do título de Mestre em Ciências, com ênfase em Recursos Genéticos Vegetais.
Orientador: Prof. Dr. Miguel Pedro Guerra

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Micropropagação, fidelidade genotípica e diversidade genética de cultivares e variedades crioulas de *Arracacia xanthorrhiza* Bancr. (“mandioquinha-salsa”)

O presente trabalho em nível de mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de mestre em Ciências com ênfase em Recursos Genéticos Vegetais.

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Dedico a todos os jovens cientistas e educadores que buscam no conhecimento ferramentas de emancipação e justiça social.

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“Las culturas indígenas de las tierras altas del centro de Colombia desaparecieron [sic], como ya se ha dicho, desde hace mucho tiempo, dejando como herencia la arracacha, a las poblaciones mestizas y blancas que ahora habitan éstas áreas. Solamente en los remotos bordes de las montañas (en sus límites con las tierras bajas) han persistido algunos aborígenes [sic], empujados al aislamiento, lejos de sus centros originales, por la presión de los modernos colonizadores blancos”. (HODGE, W. H., *La arracacha comestible*, 1949).

RESUMO

A mandioquinha-salsa (*Arracacia xanthorrhiza* Bancr.) é uma raiz tuberosa de grande valor nutricional e cultural, sendo uma importante fonte de renda para agricultores familiares em áreas úmidas de altitude ao longo das Américas do Sul e Central. Apesar disso, conta com poucos investimentos no aprimoramento de tecnologias propagativas. A dissertação a seguir é uma abordagem de ciências aplicadas com o objetivo de contribuir para o desenvolvimento de um protocolo de micropropagação para subsidiar as áreas de pesquisa e a produção de mudas de alto valor genético e qualidade sanitária em larga escala. Na seção introdutória, um cenário geral da importância da espécie e as consequências de seus sistemas de propagação *in vivo* são descritas e, na seção 2, é feita uma revisão geral da literatura produzida sobre a micropropagação de *A. xanthorrhiza*. O capítulo I é dedicado às soluções biotecnológicas para a contaminação bacteriana no estabelecimento *in vitro* e a rápida senescência, juntamente a testes com citocininas. Antibióticos de amplo espectro foram testados na introdução *in vitro*, nitrato de prata (0, 5, 15 e 45 μM) foi suplementado ao meio de cultura e morfogênese foi avaliada sub o cultivo de diferentes citocininas (BAP, KIN e mT) nas dosagens de 0, 1, 3 e 9 μM. A suplementação de ampicilina a 100 mg L-1 permitiu o estabelecimento asséptico de 70% das culturas. O $AgNO_3$ a 26 μM promove emissão de brotos e folhas e diminui o desfolhamento das plantas, potencializando a multiplicação e permitindo maior plasticidade no intervalo de subcultivo. O uso de mT a 1 μM saturou a relação de dose-resposta das plantas na emissão de folhas e brotos (3,5 brotos e 7,2 folhas por planta), apresentando três vezes mais atividade biológica e menores efeitos de inibição sobre o enraizamento que a BAP. No capítulo II foi investigado o comportamento morfogenético de plantas de *A. xanthorrhiza* cultivadas em sistemas de troca gasosa passiva (GE) e biorreatores de imersão temporária (TI) de forma comparativa aos ambientes convencionais *in agar* e herméticos. O desenvolvimento das plantas foi avaliado sob diferentes intensidades de GE (0, 9,87, 13,09, 15,58 e 81,35 GE/dia) e foram comparados meio semissólido e líquido sobre substrato de vermiculita, com e sem GE, assim como em TI RITA® e TI com frascos duplos. A densidade, morfologia e comportamento de estômatos foram observadas por diafanização e microscopia eletrônica de varredura. As plantas GE e TI não apresentaram diferenças significativas dos ambientes de cultura semissólidos e herméticos para emissões de brotações e folhas. A fase de enraizamento e aclimatização ocorreu na presença de vermiculita e a hiperidricidade foi observada apenas nas plantas oriundas de TI (8,33%), que apresentaram massa significativamente maior por planta, mas com o teor de massa seca igual a todos os tratamentos, concluindo-se que GE e TI não promoveram comportamento autotrófico. No entanto, GE e TI permitiram uma maior frequência de estômatos funcionais e com morfologia normal, quando comparados aqueles observados em condições herméticas de cultivo. Por fim, as conclusões de maior relevância as e perspectivas de investigação são descritas na seção 5.

Palavras-chave: Batata-baroa, ampicilina, AgNO3, meta-topolina, estômatos.

ABSTRACT

The arracacha (*Arracacia xanthorrhiza* Bancr.) is a tuberous root of great nutritional and cultural value and represents an important source of income for family farmers in humid highlands along the South and Central Americas. Nevertheless, this crop still receives few investments to enhance its propagative technologies. Therefore, the following thesis is an applied sciences approach aiming to contribute to the efforts towards a high-yield micropropagation protocol, focusing on solutions for the mainly observed problems in the tissue culture of the specie. In the introductory section, a general scenario of the specie importance and the consequences of its *in vivo* propagation systems were outlined, following to a general review of the literature produced over *A. xanthorrhiza* micropropagation, in the section 2. The chapter I is dedicated to technological solutions for severe bacterial contamination on *in vitro* establishment and rapid senescence, along with tests over alternative cytokinins. Wide spectrum antibiotics were tested at *in vitro* introduction, silver nitrate (0, 5, 15 and 45 μM) was supplemented to the culture medium to restrain the action of growth inhibitors and the effects over plants morphogenesis of three aromatic cytokinins (BAP, KIN and mT) in different dosages (0, 1, 3 and 9 μM) were evaluated. The ampicillin supplementation at 100 mg L-1 dosage allowed 70% of aseptic established cultures. The $AgNO_3$ at the dosage of 26 μM promotes new sprouts and shoots along with a decrease of the plants' defoliation, enhancing the multiplication factors and allowing more plasticity in the period to subcultive the plants. The use of mT at 1 μM saturated the dose-response pattern of the plants for sprouts and shoot emissions (3.5 sprouts and 7.2 leaves per plant), showing 3 times more biological activity and lower inhibition effects over rooting than the commonly used BAP. In the chapter II are presented the investigations over the morphogenetic behavior of *A. xanthorrhiza* plants cultivated under passive gas exchange (GE) and temporary immersion bioreactors systems (TI) in a comparative way to the classic *in agar* and hermetic propagation environments. Plants development was evaluated under different GE intensities (0, 9.87, 13.09, 15.58 and 81.35 GE/day) and comparisons were performed between semisolid and liquid medium over vermiculite substrate, both with and without GE, as so as in TI RITA® and TI with twin flasks. The stomata densities, morphology and behavior were also observed though diaphanization and electron scanning microscopy. The GE and TI plants had no significant differences to semisolid and hermetic culture environments for sprouts and shoots emissions. Rooting was only induced under vermiculite presence and hyperhydricity occurred only on TI plants (8.33%), which had significant higher mass per plant, but the dry mass content was statistically equal in all treatments, indicating that GE and TI did not promote an autotrophic behavior. Nonetheless, GE and TI led to a higher frequency of normal morphology and functional stomata, than those observed in hermetic culture conditions, that had predominance of opened, elevated and circular shape stomata. Finally, the remarkable conclusions and investigations perspectives are described on the section 5.

Keywords: Peruvian carrot, ampicillin, AgNO3, meta-topolin, stomata.

RESUMEN

La arracacha (*Arracacia xanthorrhiza* Bancr.) es una raíz tuberosa de gran valor nutricional y cultural y representa una importante fuente de ingresos para los agricultores familiares en zonas húmedas de altitud de Latinoamérica, pero que carece de inversiones en tecnologías de propagación. Esta tesis es un enfoque de ciencias aplicadas con el objetivo de contribuir para el desarrollo de un protocolo de micropropagación de alto rendimiento, buscando soluciones a los principales problemas observados en el cultivo de tejidos de la especie. En la introducción se describió la importancia de la especie y las consecuencias de sus sistemas de propagación *in vivo*, continuando, en la sección 2, con una revisión de la literatura producida sobre su micropropagación. El capítulo I fue dedicado a las soluciones tecnológicas para la contaminación bacteriana en el establecimiento *in vitro* y la rápida senescencia, junto con pruebas de citoquininas. Se probaron antibióticos de amplio espectro en la introducción *in vitro*, se suplementó el medio de cultivo con nitrato de plata (0, 5, 15 y 45 µM) y se evaluó la morfogénesis bajo el cultivo en diferentes citoquininas (BAP, KIN y mT) en las dosis de 0, 1, 3 y 9 µM. La suplementación de ampicilina a 100 mg L-1 permitió el establecimiento aséptico del 70% de los cultivos. El $AgNO_3$ (26 µM) promovió la emisión de brotes y hojas y he reducido la defoliación de las plantas, potenciando la multiplicación y permitiendo una mayor plasticidad en el tiempo entre los subcultivos. El uso de 1 µM mT saturó la relación dosis-respuesta de las plantas en la emisión de hojas y brotes (3,5 brotes y 7,2 hojas por planta), presentando 3 veces más actividad biológica y menos efectos de inhibición del enraizamiento que BAP. En el capítulo II, se investigó el comportamiento morfogenético de las plantas de *A. xanthorrhiza* cultivadas en sistemas pasivos de intercambio de gases (GE) y biorreactores de inmersión temporal (TI) de manera comparativa con los ambientes *in agar* y herméticos. Se evaluó el desarrollo de las plantas bajo diferentes intensidades de GE (0, 9.87, 13.09, 15.58 y 81.35 GE / día) y se compararon los medios semisólidos, liquido sobre sustrato de vermiculita, ambos con y sin GE, así como con el cultivo en TI RITA® y TI con dos matraces. La densidad, la morfología y el comportamiento de las estomas fueran observadas mediante diafanización y microscopía electrónica de barredura. Las plantas de GE y TI no mostraron diferencias significativas con los ambientes de cultivo semisólido y hermético para las emisiones de brotes y hojas. El enraizamiento solo se indujo en la presencia de vermiculita y la hiperhidricidad ocurrió solamente en plantas de TI (8,33%), las cuales presentaran mayor masa fresca por planta, pero con igual contenido de masa seca a los demás tratamientos, concluyendo que la GE e TI no promovieron el autotrofismo. Sin embargo, GE y TI presentarán mayor frecuencia de estomas funcionales y con morfología normal, en comparación con los observados en condiciones herméticas de cultivo. Finalmente, las principales conclusiones y perspectivas de investigación son descritas en la sección 5.

Keywords: apio criollo, ampicillin, AgNO3, meta-topolin, stomata.

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LISTA DE ABREVIATURAS, SIGLAS E UNIDADES DE MEDIDA

%: porcentagem

ø: diâmetro

º GL: graus Gay Lussac

º C: graus Celsius

µM: micromol

AC: *activated charcoal* (carvão ativado)

Ag: prata

a.k.a.: *also know as* (também conhecido como)

AG_3: ácido giberélico

$AgNO_3$: nitrato de prata

AIA: ácido-indolacético

AIB: ácido-indolbutírico

ALV: *Arracacha latent vírus*

AmoV: *Arracacha mottle virus*

ANA: 1-ácido-naftalenoacético

ANOVA: *analysis of variance* (análise de variância)

ASA: cultivar de mandiquinha- salsa Amarela de Senador Amaral

AVA: *Arracacha virus A*

AVB: *Arracacha virus B*

AVY: *Arracacha virus Y*

B5: formulação Salina proposta por Gamborg, Miller & Ojima (1968)

BAP: 6-benzilaminopurina

BM: *basal culture medium* (meio de cultura basal)

BMS: meio de cultura basal suplementado com vitaminas STABA

Ca: cálcio

CGIAR: Consultative Group on International Agricultural Research

CIP: *International Potato Center*

cm: centímetro

Co: cobalto

CO_2: dióxido de carbono

CORPOICA: *Corporación Colombiana de Investigación Agropecuaria*

Cu: cobre

CV%: coeficiente de variação

DNA: *Deoxyribonucleic acid* (ácido desoxirribonucleico)

EMBRAPA: Empresa Brasileira de Pesquisa Agropecuária

FEDEAGRO: *Confederación de Asociaciones de Productores Agropecuarios de Venezuela*

g: grama

GA_3: Gibberellic acid (ácido giberélico)

GE: gas exchange (troca gasosa)

ha: hectare

INIA: *Instituto Nacional de Innovación Agraria del Perú*

INIAF: *Instituto Nacional de Innovación Agropecuaria y Forestal*

INIAP: *Instituto Nacional de Investigaciones Agropecuarias de Ecuador*

KIN: N6-furfuriladenina

L: litro

LED: *light emitting diode* (diodo emissor de luz)

LQ: tratamento com meio de cultura líquido

LQ-GE: tratamento com meio de cultura líquido em frasco com troca gasosa

LQ-H: tratamento com meio de cultura líquido em frasco hermético

m: metro

mg: miligrama

Mg: magnésio

min.: minuto

ml: mililitro

mm: milímetro

mM: milimol

MS: Formulação Salina proposta por Murashige & Skoog (1962)

mT: [6-(3-hidroxibenzilamino)purina], ou meta-topolina

nº: número

n.: número

NAA: 1-Naphthaleneacetic acid

ng: nanograma

PBRSV-A: *Potato black ringspot virus - arracacha*

PCR: *polymerase chain reaction* (Reação em cadeia da polimerase)

PVS: *Potato vírus S*

R^2: coeficiente de determinação

RCBD: *randomized complete block design* (delineamento em blocos completos casualizados)

SEM: scanning electron microscopy (microscopia eletrônica de varredura)

SS: tratamento com meio de cultura semissólido

SS-GE: tratamento com meio de cultura semissólido em frasco com troca gasosa

SS-H: tratamento com meio de cultura semissólido em frasco hermético

STABA: formulação de aminoácidos proposta por Staba (1969)

s.u.: *sample unit* (unidade amostral)

TI: *temporary immersion bioreactors systems* (biorreator de imersão temporária)

TI-RITA: biorreator de imersão temporária RITA®

TI-TF: biorreator de imersão temporária com frasco duplo (*twin flask)*

ton: tonelada

UFV: Universidade Federal de Viçosa

ULA: *Universidade de los Andes*

UNALM: *Universidade Nacional Agrária de La Molina*

UNC: *Universidad Nacional de Cajamarca*

UNSAAC: *Universidad Nacional de San Antonio Abad del Cusco*

UNSCH: *Universidad Nacional de San Cristóbal de Huamanga*

USA: *United States of America* (Estados Unidos da América)

UV: radiação ultravioleta

v.: volume

w.: weghit (peso)

SUMÁRIO

1 INTRODUÇÃO

1.1 A ESPÉCIE E SUA IMPORTÂNCIA SOCIOECONÔMICA

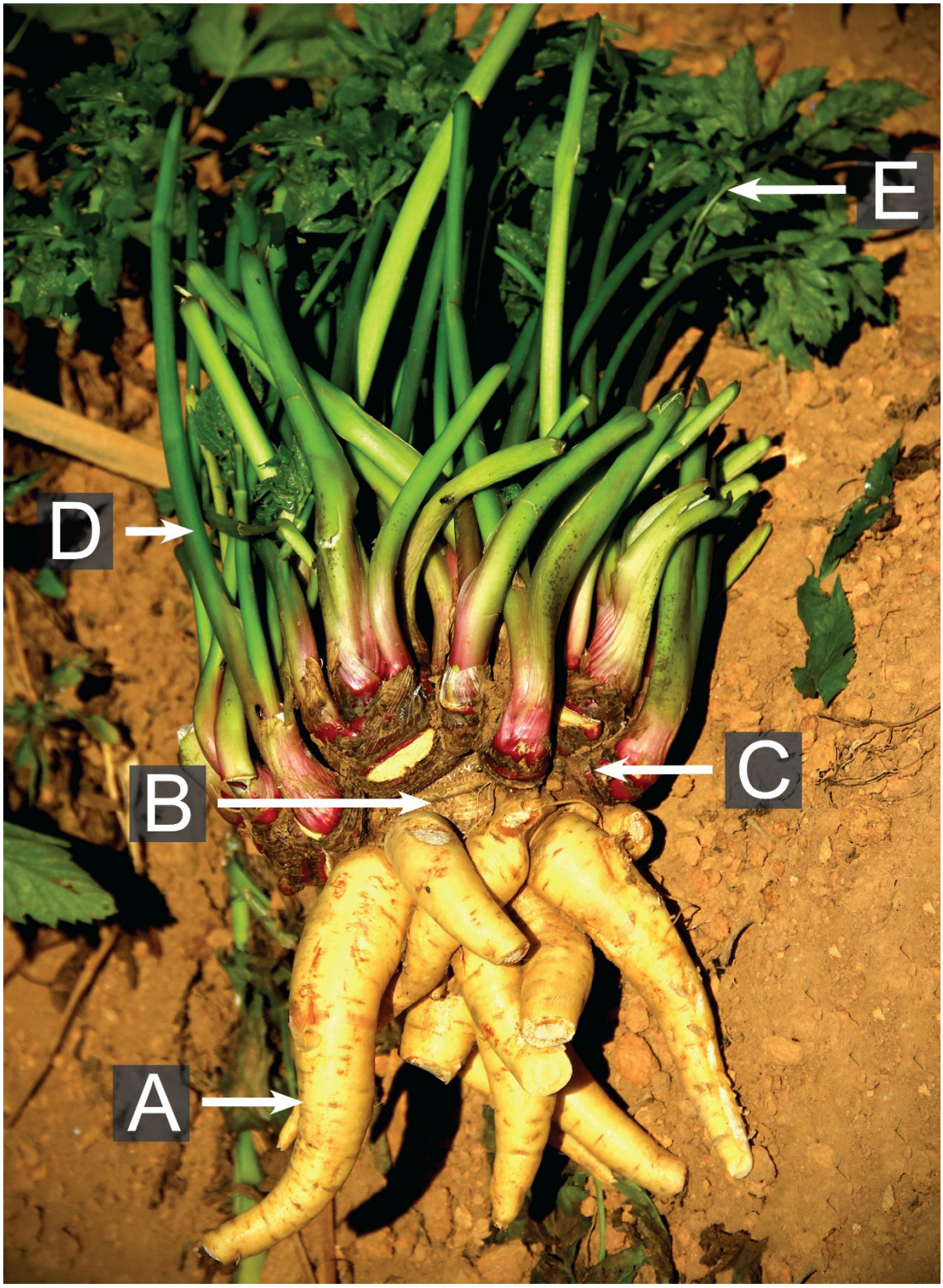
Arracacia xanthorrhiza Brancr., vernaculamente referida como "mandioquinha-salsa", mas também conhecida regionalmente como mandioquinha, batata-baroa, batata-salsa ou batata-aipo, é uma Apiaceae perene, de raízes tuberosas (Figuras 1 e 2), originária dos vales andinos, sendo provavelmente uma das primeiras plantas domesticadas na América do Sul, ainda na época pré-incaica (BERMEJO & LEÓN, 1994; HERRERA & YACOVLEFF, 1935; HODGE, 1954).

É a única espécie cultivada dentre as 30 espécies de seu gênero (MATHIAS & CONSTANCE, 1962; KNUDSEN, ØRTING & SØRENSEN, 2006), bem como a única representante de sua família botânica domesticada no novo mundo e sob propagação vegetativa (MORILLO, KNUDSEN & SÉCOND, 2017; MORILLO & SÉCOND, 2017).

As suas raízes são valorizadas pela alta plasticidade gastronômica, com aroma, cor e textura particulares que lhe conferem uma grande diversidade de pratos típicos em que são empregadas. Atuam como fonte de amidos de alta digestibilidade (80% da massa fresca), especialmente indicadas a enfermos e crianças. Também fornecem doses elevadas de cálcio, ferro, magnésio, fósforo, ácido ascórbico, β-carotenos, riboflavina, tiamina, niacina e piridoxina (SANTOS *et al*., 1993). Também são livres de efeitos anti-nutricionais existentes em outras raízes e tubérculos americanos (HERMANN, 1997), como no caso dos alcaloides da batata (*Solanum tuberosum*) e o cianeto da mandioca (*Manihot esculenta*).

Apesar de suas propriedades notáveis, a mandioquinha-salsa permaneceu como cultivo restrito ao autoconsumo de populações tradicionais das regiões andinas mesmo após séculos da invasão europeia, tendo seu cultivo expandido aos mercados somente no século XX, a partir da Colômbia (HODGE, 1954). Dentro do contexto de modernização dos países andinos e o êxodo das populações rurais para formar a força de trabalho nas áreas urbanas, o costume indígena de consumo da planta foi disseminado em áreas urbanas ao longo da América do Sul.

Figura 1 - Planta de *Arracacia xanthorrhiza* cv. 'BRS Acarijó 56' cultivada para produção de mudas: A) raízes tuberosas; B) caule-central; C) pseudocaule ou cormelo; D) pecíolo foliar; E) limbo foliar.



Fonte: acervo pessoal.

Figura 2 - Acessos do banco de germoplasma do *Instituto Nacional de Innovación Agropecuaria y Forestal* – INIAF (Cochabamba - Bolívia) que representam a variação fenotípica das raízes e cormelos de *A. xanthorrhiza*. De modo geral, a coloração do amido (matizes entre o branco e o amarelo) e do cilindro vascular das raízes (matizes entre o branco, amarelo, laranja, roxo e azul) são utilizados para definição de suas formas hortícolas.

Fonte: adaptado de INIAF, 2010

Atualmente é cultivada em áreas úmidas e de altitude (600 a 3500 msnm) em pelo menos 10 países latino-americanos – Brasil, Bolívia, Chile, Colômbia, Costa Rica, Cuba, Equador, Peru, Porto Rico e Venezuela (HERMANN, 1997) – havendo ainda registros de pequenos cultivos na República Dominicana, Haiti, Jamaica, Panamá, Argentina e até mesmo fora das Américas, no Sri Lanka, Burundi, Ruanda, Moçambique e Nova Zelândia.

Neste contexto, estima-se que 80 a 100 milhões de pessoas tenham a planta como parte de sua dieta, ao menos de forma ocasional (HERMANN, 1997), consumindo sobretudo as raízes tuberosas e, em alguns casos, mais restritos às populações tradicionais caribenhas, o nó central do caule central. Agricultores familiares do Sul do Brasil utilizam também as folhas no pós-colheita como forragem animal, rica em proteínas para o gado.

Apesar da recente emergência, e provável futura predominância, do seu cultivo em áreas de até 100 a 1.000 hectares no sul e sudeste do Brasil, a mandioquinha-salsa ainda tem como realidade produtiva mais comum, aqui e no restante da América Latina, as condições de cultivo comuns a agricultura familiar, realizada em pequenas áreas, geralmente de 0,5 a 3,0 ha, com uso intensivo de mão-de-obra no preparo das mudas, condicionamento do solo, plantio, tratos culturais e colheita.

Assim como ocorre em outras culturas secundárias para o mercado, não existem compilações atualizadas sobre dados globais de produção e consumo de mandioquinha-salsa, dispondo-se apenas de informações fornecidas por pesquisadores de instituições de pesquisa, extensão rural e universidades (tabela 1). Desconsiderando a Bolívia, país para o qual não se encontraram dados de produção recentes, estima-se que cerca de 25 mil hectares da cultura sejam plantados anualmente, apenas na América do Sul.

Tabela 1 - Área plantada (ha), produção anual e produtividade de mandioquinha-salsa nos principais países produtores, exceto Bolívia:

País	Ano de referência	Fonte dos Dados	Área plantada (ha)	Produção anual (ton/ano)	Produtividade (ton/ha)
Brasil	2017	Embrapa	8.168	109.227	13,4
Colômbia	2017	Corpoica	8.870	91.866	10,5
Equador	Não informado	Univ. Quito	500	1.900	3,8
Perú	Não informado	CIP/CGIAR	4.000	24.000	6,0
Venezuela	2015	Fedeagro	4.000	60.000	15,0

Fonte: dados fornecidos por pesquisadores participantes do I Encuentro Latinoamericano de Arracacha (Pouso Alegre-MG, 2018).

A Colômbia e o Brasil destacam-se como os principais produtores de raízes da planta, tendo o primeiro a maior área plantada e produção anual. Desde sua introdução no Brasil, no início do século XX, a partir de duas variedades doadas pelo governo colombiano (JARAMILLO ARANGO, 1986), a cultura espalhou-se por territórios da agricultura familiar no Sul e Sudeste do país e ganhou crescente valorização no mercado.

Nas últimas três décadas, a Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA) tem trabalhado com melhoramento da espécie e, desde então, segue disponibilizando cultivares com maior precocidade (redução do ciclo habitual de 10 a 12 meses para 7 a 10 meses) e produtividade, junto à capacitação técnica de extensionistas e agricultores multiplicadores, através de programas de transferência de tecnologia. Os trabalhos conjuntos da EMBRAPA com as agências de extensão rural estaduais impulsionaram a cultura, que se tornou uma das principais fontes de renda de agricultores familiares em regiões como o Sul de Minas e a Serra da Mantiqueira em Minas Gerais e na Encosta da Serra Geral, em Santa Catarina.

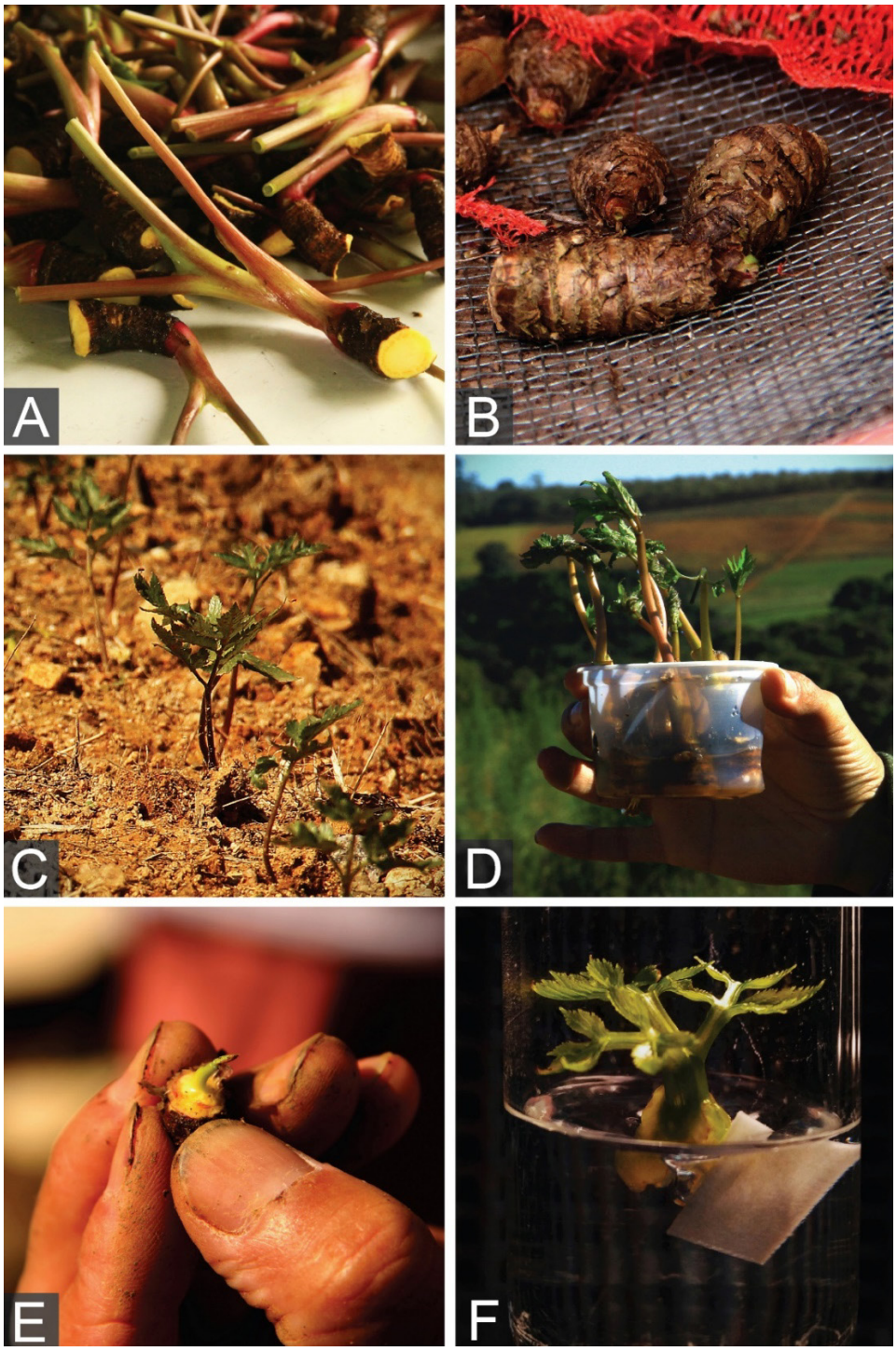
1.2 LIMITAÇÕES NAS TECNOLOGIAS DE PROPAGAÇÃO

Apesar dos esforços de entidades públicas para viabilizar a certificação da produção de mudas e distribuição de materiais genéticos com boas qualidades morfológicas, fisiológicas e sanitárias (MADEIRA & CARVALHO, 2016; MADEIRA *et* al., 2017), a cultura ainda recebe incentivos insuficientes das agencias públicas para o uso de novas tecnologias que poderiam melhorar a qualidade do material propagativo.

De forma majoritária nos campos de produção de raízes de mandioquinha-salsa, os materiais propagativos são obtidos através de cormelos (comumente chamados de perfilhos, rebentos ou propágulos), isolados no momento da colheita e que são mais tarde (períodos que, geralmente, seguem de 0 a 21 dias) plantados diretamente a campo ou armazenados e/ou pre-brotados e vendidos a terceiros, sem muitos critérios de estratificação ou seleção por tamanho e sanidade, ou cuidados com assepsia dos propágulos (figura 3).

Figura 3 - Propagação vegetativa em *A xanthorrhiza*: A) cormelos recém destacados da planta e prontos para o plantio, após desinfestação e corte em bisel; B) cormelos armazenados em sacas de rafia por 21 dias, aguardando o momento de plantio; C) mudas em pré-brotação e enraizamento por 45 dias em canteiro dedicado; D) mudas em pré-brotação por 21 dias em pote plástico com água; E) ápice caulinar utilizado como explante para introdução da planta *in vitro*; F) plântula estabelecida *in vitro* para micropropagação.

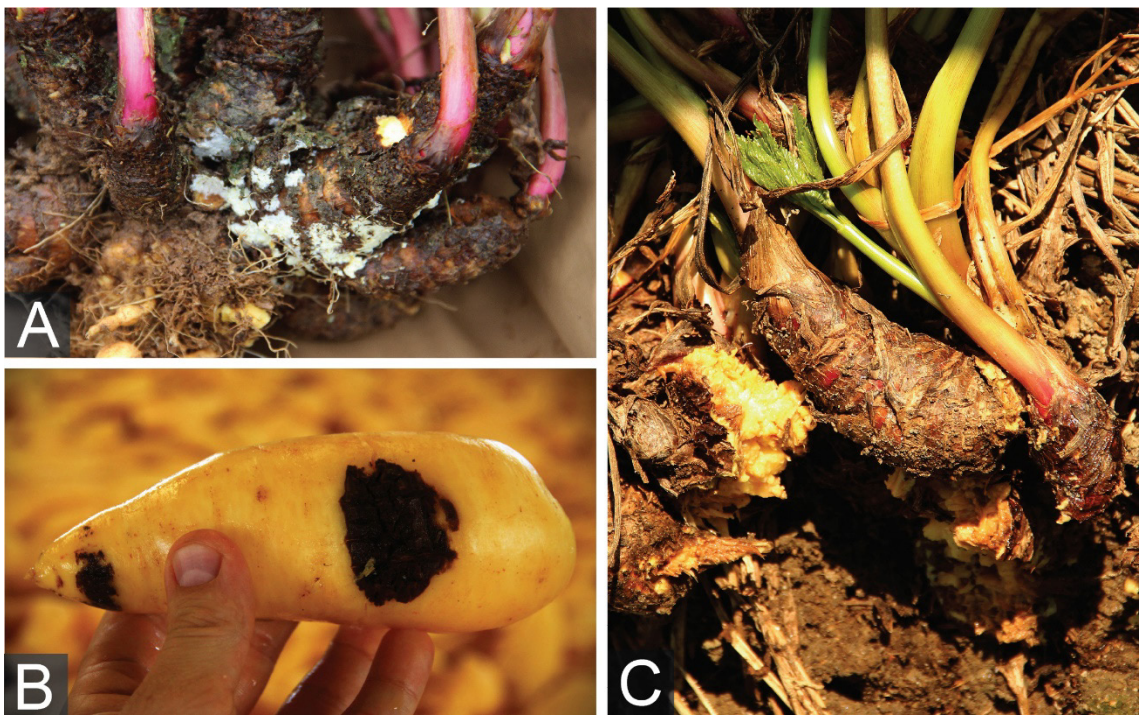
Fonte: acervo pessoal.



A desuniformidade fenológica e da quantidade de reservas disponíveis nos propágulos, a perda de vigor e estiolamento durante o armazenamento inadequado e a ausência comum de práticas de desinfestação e pré-enraizamento dos propágulos antes de seu transplante a campo, contribuem para falhas em áreas de produção que chegam a alcançar até 30% do estande de plantas planejado, limitando enormemente a produtividade das áreas de cultivo desde o momento de sua implantação (SANTOS & CARMO, 1998; MADEIRA *et* al., 2017).

Propágulos oriundos diretamente de áreas comerciais, nas quais não se faz possível um controle fitossanitário e isolamento ideais à produção de propágulos (como no caso de jardins clonais e campos de mudas), e nas quais, geralmente, não há uma cultura de boas práticas de assepsia dos materiais propagativos, como a lavagem em solução de 0,1% hipoclorito de sódio (MADEIRA *et* al., 2017), acabam por promover a dispersão de doenças de difícil erradicação e pragas que comprometem seriamente o desenvolvimento das plantas (figura 4).

Figura 4 - Doenças da cultura da mandioquinha-salsa mais comumente transmissíveis através de materiais propagativos oriundos de campos de produção de raízes: A) cormelos atacados por mofo-branco (*Sclerotinia* spp.); B) raiz tuberosa danificada por nematoide-das-lesões (*Pratylenchus* spp.); C) podridão bacteriana (*Erwinia* spp.), alastrando-se das raízes até a parte aérea da planta.



Fonte: acervo pessoal.

Dentre as mais preocupantes, destacam-se as nematoses (*Meloidogyne* spp. e *Pratylenchus* spp.), podridões sistêmicas fúngicas (*Sclerotinia sclerotiorum* e *Sclerotium rolfsii*) e bacterianas (*Erwinia* spp.) (HENZ, 2002).

Pragas de alto impacto para a cultura também podem ser disseminadas por material de propagação, tais como ácaros (*Tetranychus* spp.), afídeos (*Hiadaphis foeniculi*, *Anuraphis* sp. e *Aphis* sp.) e coleópteros (*Conotrachelus cristatus*), os quais, por estarem alojados nos tecidos internos ou externos dos cormelos e pecíolos reduzem o estabelecimento das plantas e provocam ferimentos propícios para infecção por patógenos.

Ao mesmo tempo, o contínuo acúmulo de viroses, inerente a espécies de propagação vegetativa e a este conjunto de práticas de propagação, é especulado como possível fator de degeneração da capacidade produtiva destes materiais genéticos (MADEIRA, 2004).

O grau de disseminação e os efeitos fisiológicos das viroses sobre a robustez da mandioquinha-salsa ainda é incerto, mas sete viroses incidentes já foram identificadas – *Arracacha latent vírus* - ALV (LIZÁRRAGA, 1997), *Potato vírus S* – PVS (DE SOUZA *et al.*, 2017), *Arracacha virus B* – AVB (KENTEN & JONES, 1979), *Arracacha virus A* - AVA (JONES & KENTEN, 1978), *Potato black ringspot virus - arracacha* - PBRSV-A (LIZÁRRAGA *et al*., 1994), *Arracacha virus Y* – AVY (LIZÁRRAGA, 1997), *Arracacha mottle virus* – AMoV (ORILIO *et al*., 2009) – algumas atacando exclusivamente a espécie e outras são, preocupantemente, compartilhadas com grandes culturas, com destaque às solanáceas como a batata e o tomate.

A equipe da Embrapa Hortaliças juntamente com a Emater-MG tem feito um valoroso esforço propositivo de uma cadeia de produção de mudas certificadas para a mandioquinha salsa, que contornam eficazmente boa parte das limitações supracitadas. Madeira *et al*. (2017) propõem o estabelecimento de jardins clonais sob cultivo protegido para cultivares, a partir dos quais, campos de mudas (ou *campo de plantas*, como define a Instrução Normativa MAPA nº 24 de 16 de dezembro de 2005 (BRASIL, 2005), doravante, IN24/2005) podem receber materiais propagativos para multiplicar a produção de mudas para comercialização geral.

Nesse modelo produtivo em duas etapas, os jardins clonais apresentariam um alto grau de controle fitossanitário, através do uso de propágulos oriundos da *cultura de tecidos* (idealmente, culturas de meristemas, já indexadas como livres de viroses), estabelecidos em substrato sanitizado, irrigados com água livre de agentes patogênicos e dispostos em estufas com telado antiafídeo e submetidos à inspeção sistemática e periódica de qualidade e sanidade. Por sua vez, os campos de mudas, seriam feitos em campo aberto, baseados nos propágulos

oriundos dos jardins clonais e na própria produção, de onde os campos de produção de raízes poderiam obter seus propágulos (MADEIRA *et* al., 2017).

No que tange à certificação, o modelo proposto requer adaptações no texto da IN24/2005 ou sua complementação por outro instrumento normativo próprio a ser redigido, visto que o regramento vigente que não preconiza a certificação de jardim clonal que não seja composto por plantas básicas ou plantas matrizes – algo não praticável no caso da mandioquinha-salsa, que é uma planta perene, mas cultivada como anual.

Também existem limitações no tocante à qualidade do material a ser utilizado para a composição dos jardins clonais. Idealmente, os mesmos poderiam ser oriundos de plantas regeneradas através de culturas de meristemas, a partir de protocolos como os desenvolvidos por Luz (1993), Madeira (2004) e Slíva, Viehmannová & Vítámvás (2010).

Entretanto, fora a própria Embrapa Hortaliças e no caso de uma única biofábrica brasileira que produz mudas para um empreendimento próprio, mudas oriundas da cultura de tecidos não estão disponíveis, em boa medida, pelo alto custo e dificuldades operacionais e lacunas dos protocolos de micropropagação disponíveis, os quais discutiremos em maior detalhe na revisão bibliográfica.

1.3 INDÍCIOS DE EROSÃO GENÉTICA

Se por um lado os genótipos melhorados trazem incentivos ao cultivo da espécie pela maior produtividade e rentabilidade, por outro, deslocam algumas variedades tradicionais do contexto produtivo. Plantas de variedades com raízes brancas ou com matizes arroxeadas têm persistido apenas como gêneros de autoconsumo, em comunidades ermas (sobretudo, nos países andinos), pois não se adequam às demandas de forma e coloração preferidas pelo mercado, no qual consumidores buscam raízes curtas, de maior diâmetro e amido de coloração amarela.

A agrobiodiversidade que persistiu ao largo das dinâmicas de mercado permanece pouco conhecida pelos entes governamentais e entidades conservacionistas, impedindo que se dimensione adequadamente o nível de erosão genética existente na cultura.

Os bancos de germoplasma (tabela 2) que teriam o papel de resguardo das variedades tradicionais são escassos e sofrem com a falta de investimento e condições técnicas para uma atuação eficaz. Os números de acessos de *A. xanthorrhiza* reportados por estas instituições devem ser tomados com ressalvas, pois a maior parte delas possui dados desatualizados e raramente as informações estão disponíveis *on-line*, de forma que, em boa parte dos casos,

conta-se apenas com comunicações pessoais de pesquisadores com acesso direto aos bancos de germoplasma e seus operadores para a aferição da integridade atual das coleções.

Coleções das universidades estatais peruanas estão muito erodidas desde o levantamento feito por Hermann (1997), com perdas de mais de 50% dos acessos por falta de investimentos, dificuldades de manejo e efeitos das mudanças climáticas (Ivan Manrique/CIP-CGIAR, comunicação pessoal).

Tabela 2 - Bancos de germoplasma com acessos de variedades cultivadas de *A. xanthorrhiza*

Instituição	País	Ano de Referência	Nº de Acessos	País de Origem dos Acessos	Referência
Instituto Nacional de Innovacíon Agraria (INIA)	Peru	2018	145	Peru	Ivan Manrique, comunicação pessoal
Corporación Colombiana de Investigación Agropecuaria (CORPOICA)	Colômbia	2010	123	Colômbia	VALENCIA RAMÍREZ *et al*., 2010
Universidad Nacional de Cajamarca (UNC)	Peru	2018	122	Peru	Ivan Manrique, comunicação pessoal
Instituto Nacional de Investigaciones Agropecuarias (INIAP)	Equador	2018	109	Equador	Eduardo Morillo, comunicação pessoal
Universidad Nacional de San Antonio Abad del Cusco (UNSAAC)	Peru	1997	104	Peru	HERMANN, 1997
Universidade Federal de Viçosa (UFV)	Brasil	2018	6	Brasil	BHG-UFV, 2018
Centro Internacional de la Papa (CIP/CGIAR)	Peru	2017	42	Brasil, Colômbia, Equador, Peru, Bolivia e Chile	CIP Genebank
Centro Nacional de Pesquisas em Hortaliças da (EMBRAPA HORTALIÇAS)	Brasil	2018	28	Brasil e Equador	Nuno Madeira, comunicação pessoal
Universidade de los Andes (ULA)	Venezuela	2014	24	Venezuela e Peru	Ramon Jaimez, comunicação pessoal
Instituto Nacional de Innovación Agropecuaria y Forestal (INIAF)	Bolívia	2010	21	Bolivia	INIAF, 2010
Universidad Nacional de San Cristóbal de Huamanga (UNSCH)	Peru	2018	20	Peru	Ivan Manrique, comunicação pessoal
Universidade Nacional Agrária de La Molina (UNALM)	Peru	2018	1	Peru	Ivan Manrique, comunicação pessoal
Total de acessos em bancos de germoplasma			***745***		

Fonte: dados fornecidos por pesquisadores participantes do I Encuentro Latinoamericano de Arracacha (Pouso Alegre-MG, 2018).

Mesmo instituições com aporte de recursos têm perdido acessos ao longo do tempo por causas similares, como no caso do *Centro Internacional de la Papa* – CIP-CGIAR, que já contou com 66 acessos e hoje possui apenas 42 destes.

A Embrapa Hortaliças, que chegou a ter mais de 2000 acessos (SANTOS, 1997), a maioria, porém oriunda de sementes botânicas que em geral apresentam baixo vigor. Em 2002, contava com apenas 139 clones (CHARCHAR, SANTOS & MOITA, 2007), passando para 60 acessos no ano de 2014 e para os atuais para 28 acessos, mantidos em condições de campo. A maior causa de perdas de acessos ocorre pelo efeito de intempéries e mudanças climáticas especialmente por que o Distrito Federal está no limite de aptidão climática para a cultura (Nuno Madeira, Embrapa Hortaliças, comunicação pessoal).

Estas informações denotam a fragilidade do status atual dos bancos de germoplasma a campo e a um investimento de recursos aquém do necessário nos meios institucionais de pesquisa e extensão, com poucos editais e provisionamentos destinados à espécie e seu cultivo. O resguardo de clones *in vitro* é feito apenas pelo CIP-CGIAR, em cerca de um quarto dos acessos.

A possibilidade de resguardo em cópias complementares e intercâmbio de germoplasma é limitada por problemas fitossanitários com viroses, tratados internacionais (CDB, 2005; FAO, 2009) e legislações nacionais de proteção à biodiversidade.

1.4 PROBLEMA DE PESQUISA E JUSTIFICATIVA

Os propágulos utilizados na cultura da mandioquinha-salsa, feitos a partir de técnicas horticulturais convencionais, reduzem a uniformidade das plantas a campo, aumentam o tempo do ciclo produtivo, requerem grande quantidade de mão-de-obra e tendem a transportar uma série de patógenos e pragas que geram a degenerescência gradual dos materiais genéticos.

Ao mesmo tempo, a diversidade genética da cultura encontra-se ameaçada pela perda de áreas cultivadas com variedades tradicionais e os bancos de germoplasma seguem sofrendo erosões genéticas pela manutenção dos acessos, majoritariamente, sob condições de campo.

Para além das perdas produtivas, este contexto reduz a atratividade econômica da atividade pela perda da resiliência ecológica das formas cultivadas da espécie – variáveis que se somam ao quadro geral de risco à segurança alimentar e à reprodução social de diversos agricultores familiares ao longo da América Latina.

Uma parcela significativa destes entraves na propagação e conservação da agrobiodiversidade de *A. xanthorrhiza* podem ser contornados pela pesquisa e desenvolvimento de biotecnologias, notadamente no uso de ferramentas de cultura de tecidos voltadas à propagação massal e clonal (micropropagação) de genótipos elite, mantendo a qualidade genética, fisiológica e fitossanitária propágulos produzidos.

Protocolos de micropropagação da espécie podem atuar como referencial tecnológico para a instalação gradual de laboratórios comerciais (biofábricas), capazes de fornecer materiais básicos para jardins clonais, comercializá-las diretamente com campos de mudas ou até mesmo de forma direta com agricultores envolvidos na produção de raízes.

Esta estratégia visa o aumento do vigor e qualidade genética do material propagativo, reduzindo obstáculos às de sanidade, bem como, gerando ganhos diretos em índices de produtividade.

Já são reportados protocolos de multiplicação de culturas organogenéticas de *A. xanthorrhiza* com boa eficiência multiplicativa (MADEIRA, 2004; MATOS *et al*., 2015). Contudo, conforme se discutirá adiante, o número de fatores multiplicativos testados na literatura dedicada é limitado.

A micropropagação também pode atuar na contenção da erosão genética em bancos de germoplasma pela manutenção de acessos *in vitro* (PANTA *et al*., 2009; BENSON *et al*., 2011) e, de modo indireto, pode promover a conservação pelo uso, multiplicando materiais e repatriando propágulos de variedades crioulas deslocadas das dinâmicas de mercado e/ou restritas a contextos locais ou aos próprios bancos de germoplasma, por exemplo.

Por fim, protocolos de micropropagação de alta eficácia multiplicativa são fundamentais ao desenvolvimento e à aplicação de outras biotecnologias que subsidiam estudos de ciências básicas e aplicadas na contínua domesticação e conservação dos recursos genéticos da espécie.

Assim, desenvolveu-se revisão do estado da arte das tecnologias voltadas à micropropagação de *A. xanthorrhiza* ("Revisão bibliográfica") e uma estrutura de pesquisa, dividida em duas etapas (capítulos) de exposição: I) "Novas abordagens na micropropagação de *Arracacia xanthorrhiza* Bancr. ('mandioquinha-salsa'): estabelecimento *in vitro,* redução da senescência, e balanço de reguladores de crescimento; II) Morfogênese *in vitro* de plantas de *Arracacia xanthorrhiza* Bancr. ('mandioquinha-salsa') cultivadas em diferentes intensidades de troca gasosa e biorreatores de imersão temporária". Por fim, foram sintetizadas as principais conclusões e perspectivas de investigação futuras a partir das lacunas encontradas.

1.5 OBJETIVOS

1.5.1 Objetivo Geral

Desenvolver um protocolo de micropropagação com alta eficiência multiplicativa para mandioquinha-salsa (*Arracacia xanthorrhiza* Bancr.).

1.5.2 Objetivos Específicos

- Otimizar protocolos de assepsia e introdução *in vitro* de ápices caulinares;
- Determinar a eficiência de compostos sequestradores e repressores de inibidores do crescimento vegetal *in vitro*;
- Testar os efeitos de diferentes fontes e doses de citocininas sobre a morfogênese *in vitro*;
- Comparar desenvolvimento de culturas organogenéticas sob distintos regimes de trocas gasosas passivas e em biorreatores aos sistemas clássicos de cultivo *in agar*.

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2 REVISÃO BIBLIOGRÁFICA

2.1 AVANÇOS E LACUNAS NA MICROPROPAGAÇÃO DA MANDIOQUINHA-SALSA

Os primeiros trabalhos de micropropagação de *A. xanthorrhiza* datam do final da década de 1980 e meados na década de 1990. Contudo, os registros das pesquisas ainda são relativamente escassos, algumas de difícil acesso, restritas a repositórios físicos de universidades estrangeiras ou anais de congresso, de modo que, para esta revisão, nos atentamos, sobretudo, a dissertações, teses e artigos de periódicos científicos.

O primeiro registro de um protocolo de introdução *in vitro* da espécie foi feito por Senna Neto (1990), que apontou a melhor adequação da solução salina B5 (GAMBORG, MILLER & OJIMA, 1968) para o desenvolvimento de meristemas. Esse autor estimulou a morfogênese e multiplicação *in vitro* com diferentes balanços de 6-benzilaminopurina (BAP) e ácido-naftalenoacético (ANA), encontrando resultados condizentes ao experimento clássico de Skoog & Miller (1957).

Seguindo esta linha de trabalhos, Luz (1993) alcançou melhores taxas regenerativas para os meristemas introduzidos *in vitro* utilizando BAP (0,88 μM), ANA (0,54 μM) com a adição de ácido giberélico (AG_3) para promoção de brotações. Também determinou condições de enraizamento, através da adição isolada de 0,54 μM de ANA ao meio de cultura.

O estabelecimento *in vitro* de oito acessos do banco de germoplasma do *Instituto Nacional de Investigaciones Agropecuarias* (INIAP-Equador) por meio de ápices caulinares foi feito por Cevallos Freire (1991). A referida pesquisadora utilizou meios de cultura baseados em composições salinas e vitaminas MS (MURASHIGE & SKOOG, 1962) e diferentes composições de reguladores de crescimento, que incluíam a ausência ou presença de AG_3, BAP, ANA, ácido-indolbutírico (AIB), ácido-indolacético (AIA). Apesar de concluir por uma forte dependência do genótipo à resposta morfogenética, a autora recomendou o uso de meio MS suplementado com AG_3 (0,72 μM) e AIA (0,29 μM) como o mais adequado ao desenvolvimento de parte aérea e raízes de pelo menos duas linhagens. Também observou altas taxas de contaminação bacteriana dificultando o estabelecimento das culturas.

Landázuri (1996) e Duque & Landázuri (1999) desenvolveram um protocolo de introdução *in vitro* de ápices caulinares e gemas adventícias (4 a 8 mm) que reportava até 83% de eficácia, através de três assepsias combinadas no cormo, nos brotos e no explante final, com uso de álcool etílico (75° GL), hipoclorito de sódio (2,5%) e polisorbato 20. Os autores também

testaram duas composições salinas (MS e B5), suplementadas com BAP (de 3,11 até 35,62 μM) e uma baixa concentração de ANA (0,27 μM), concluindo pela superioridade do meio MS suplementado com 24,86 μM de BAP para indução de brotações. A adição de sorbitol (4% p./v.) ao meio de cultivo permitiu ainda o estabelecimento sem repicagem por 4 meses.

O trabalho de Madeira (2004) recuperou em parte as investigações de Senna Neto (1990) e Luz (1993), comparando diferentes combinações salinas e investigando os efeitos das concentrações de BAP e AG_3 no desenvolvimento de ápices caulinares muito reduzidos (três tamanhos testados, incluindo ápices com aproximadamente 0,4 mm) de *A. xanthorrhiza*. Terminou por concluir pela maior adequação da formulação salina B5 para o desenvolvimento morfogenético das plantas e apontou a melhor eficácia organogenética no uso de uma concentração mais baixa de BAP na indução de brotações (1,33 μM) combinado à adição de 0,72 μM de AG_3 e 0,54 μM de ANA.

Um grupo de pesquisadores tchecos também desenvolveu uma proposta de protocolo para organogênese: Slíva, Viehmannová & Vítámvás (2010) testaram o desenvolvimento de meristemas em meios de cultura com sais MS à metade da concentração usual e diferentes balanços entre as concentrações de BAP e ANA. Destarte, inferiram pela melhor combinação para indução de brotações com uso de BAP a 4,44 μM e ANA a 0,54 μM.

O trabalho mais recente sobre organogênese publicado é o de Matos *et al.* (2015), no qual ápices caulinares (4 mm) de cinco cultivares foram testadas em meio B5 e MS suplementados por ANA (0,54 μM) e BAP (1,33 μM). Além de confirmar a genótipo dependência observada por Cevallos Freire (1991), os pesquisadores indicam a formulação salina B5 como maior promotora do estabelecimento e multiplicação das variedades testadas.

Como se pode perceber ao longo desta breve revisão (sintetizada na tabela 3), existem diversos desencontros entre as informações sobre qual a formulação salina mais adequada (B5 ou MS) e quais as combinações e concentrações de reguladores seriam mais eficazes à morfogênese e multiplicação.

Provavelmente estas diferenças se devem a pequenas unidades amostrais (pouco descritas na maior parte nos trabalhos), mas também a estratégias distintas de controle da morfogênese vegetal e a provável genótipo dependência da resposta às concentrações de nutrientes e reguladores de crescimento, conservadas entre os autores que testaram mais do que duas variedades.

Tabela 3 - Uso de formulações salinas, reguladores de crescimento e concentrações de trabalhos reportadas na literatura acessível como melhores combinações encontradas para o estabelecimento e multiplicação de culturas organogenéticas.

Reporte	Formulação salina	Reguladores de Crescimento e Concentração de Trabalho (µM)			
		ANA	AIA	BAP	AG3
Sena Neto (1990)	B5	0,11	-	0,44	-
Luz (1993)	B5	0,57	-	0,88	0,72
Cevallos Freire (1991)	MS	-	0,29	-	0,72
Duque & Landázuri (1999)	MS	0,27	-	24,86	-
Madeira (2004)	B5	0,54	-	1,33	0,72
Slíva *et. al* (2010)	MS	0,54	-	4,44	-
Matos *et. al* (2015)	B5	0,54	-	1,33	-

Também é notável que os testes se detenham sempre sobre os mesmos fatores da indução de multiplicação: não somente as comparações enfocam-se sobre as mesmas formulações salinas, como utilizam quase sempre as mesmas citocinina (BAP) e auxina (ANA). Não são descritos testes com supressores de inibidores do crescimento, uso de trocas gasosas ou de biorreatores no processo de micropropagação de *A. xanthorrhiza*.

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3 CAPÍTULO I – NEW APPROACHES ON MICROPROPAGATION OF *ARRACACIA XANTHORRHIZA* BANCR. ("ARRACACHA"): *IN VITRO* ESTABLISHMENT, SENESCENCE REDUCTION AND GROWTH REGULATORS BALANCE

3.1 ABSTRACT

The following paper is part of the efforts in order to develop a micropropagation protocol for *A. xanthorrhiza*, focusing in solutions for the mainly observed problems, like severe bacterial contamination on *in vitro* establishment, rapid senescence and unexplored potentials of alternative cytokinins. Wide spectrum antibiotics, embed in paper capsules, were tested at *in vitro* introduction; activated charcoal (1.75 g L^{-1}) and silver nitrate (0, 5, 15 and 45 μM) were supplemented to the culture medium in order to absorb or restrain the action of growth inhibitors, like ethylene; and the effects over plants morphogenesis of three aromatic cytokinins (BAP, KIN and mT) in different dosages (0, 1, 3 and 9 μM) where evaluated. Ampicillin at 100 mg L^{-1} dosage allowed 70% of aseptic established cultures, then validated as an effective and low-cost method for bacterial contaminants control. The use of AgNO3 at the dosage of 26 μM promoted new sprouts and shoots along decrease of the plant's defoliation, enhancing the multiplication factors and allowing more plasticity in the subculture time. The use of mT at 1 μM saturates the dose-response of the plants for sprouts and shoot emission (3.5 sprouts and 7.2 leaves per plant), showing three-folds times more biological activity and lower inhibition effects over rooting than the commonly used BAP.

Keywords: Apiaceae, mandioquinha-salsa, ampicillin, $AgNO_3$, meta-topolin.

3.2 INTRODUCTION

The arracacha (*Arracacia xanthorrhiza* Bancr.) is a tuberous root of great nutritional, cultural and economic value, representing an important source of income for family farmers in humid highlands of Brazil and the Andean and Caribbean countries. However, this crop receives few technologies enhancements, which could improve the quality of propagative material and its conservation.

Since the recognition of its gastronomic plasticity and delicate taste and aroma, the arracacha crops were expanded from the Andean indigenous communities to the urban markets

of South America as value-added merchandise (HERMANN, 1997). Modern cultivars emerged, especially in Brazil and Colombia in the decade of 1990, meanwhile landraces are still preserved in traditional communities and used as source for modern breeding since the germplasm banks in Peru, Bolivia, Ecuador, Colombia and Brazil.

Along these movements, studies on *in vitro* behavior of these varieties where developed, searching for optimal conditions for the species micropropagation. Notwithstanding the potentialities of the plant tissue culture, the *A. xanthorrhiza* culture is still mainly multiplied by isolation of cormels from field plants, a traditional technique that maintain the genetic e phenotypic desired traits, but is the main agent for the spread of fungi, bacterial and viral diseases correlated with degeneracy of the productive potential and genetic erosion in field crops and genebanks (GIORDANO *et al*.,1995; HENZ, 2002; MADEIRA, 2004).

The present work is part of the efforts in order to develop a micropropagation protocol with low-cost, technically simple and with high multiplication yield for *A. xanthorrhiza*, having a perspective that this biotechnology is a basis for research in various fields of plant science and, above all, aims at obtaining healthy plants for breeding, international germplasm exchange, genetic conservation, food and nutritional security.

Henceforward, the experiments developed are focused on the mainly observed problems in the tissue culture of the specie, like severe bacterial contamination on *in vitro* establishment, slow growth and rapid senesce, as so as the little explored cytokinins like meta-topolins.

3.3 MATERIAL AND METHODS

3.3.1 Plant material

All tests performed *in vitro* were made based on mother plants kept in greenhouse conditions. The matrices were originated from cormels of healthy and vigorous plants selected from production the fields of family farmers, in the municipality of Angelina (S 27.456.258, E 49.063.053), at Santa Catarina State, South region of Brazil.

The cultivar *Amarela de Senador Amaral* (ASA) was selected to accomplish the tests that compose the basics data to support the protocol development, due to its representativeness in the crop fields, once occupying 95% of the cultivated areas in the country (MADEIRA *et al*., 2017) and still today, being planted in more than 80% of its crops.

3.3.2 *In vitro* introduction, basal medium and growing conditions

The disinfestation procedure for *in vitro* introduction was performed by means three phases of plant tissue reduction and asepsis. Primarily, cormels with 3 cm of basal reserve tissue and 3 cm of petioles were excised from the mother plants a washed under constant water flow for 10 min. and then immersed in 70% alcohol for 3 min., followed by an immersion in sodium hypochlorite 4% (v./v.) and polysorbate-20 (1 ml L^{-1}) for 20 min. Aiming to obtain maximum vigor, establishment rate, sanity, and genetic fidelity, only apical sprouts where utilized in the process.

Thereafter, the explants were reduced to dimensions about 1 to 1.5 cm high, leaving only 5 mm of basal tissue and thenceforth sent to a laminar flow cabinet to accomplish a new disinfestation of the tissues, made by immersion in alcohol 70% for 1 min., followed by an immersion in sodium hypochlorite 2% (v./v.) and polysorbate-20 (3 drops per 100 ml) for 20 min.

Finally, the material was rinsed three times in sterile deionized water and the tissues with visual chlorine damage in the extremities were excised with a scalpel, leaving the final explant with sizes from 0.7 to 1 cm high, before the inoculation into the culture medium.

Based on the previous works of Senna Neto (1990), Luz (1993), Madeira (2004) and Matos *et al*. (2015), we used a culture medium based on B5 saline and vitamins formulation (GAMBORG, MILLER & OJIMA, 1968), supplemented with sucrose 3% (w./v.), 0.5 µM of α-Naphthaleneacetic acid (NAA), and 3 µM of 6-Benzylaminopurine (BAP), gelified with Phytagel™ 0.2% (w./v.) and pH adjusted to 5.8 with 1N KOH, before sterilization in autoclave at 121 °C for 15 min.

Each explant was inoculated in 25 mm ø essay tubes containing 10 ml of basal culture medium (BM) and incubated in growth room at 25 ± 2 °C, 60% ± 10% of relative humidity and a photoperiod of 16 hours hours under 50 µM m-2 s-1, supplied by white spectrum LED lamps (GreenPower TLED W, Philips™, USA).

3.3.3 Antibiotic testing

In order to control contaminations with associated bacteria in the newly introduced cultures, two wide spectrum antibiotics were tested: ampicillin sodium salt (100 mg L^{-1}) and

tetracycline hydrochloride (20 mg L^{-1}), following the manufacturer (Sigma-Aldrich Corporation, USA) suggestion for work concentrations.

A stock solution of each antibiotic was prepared and filter sterilized to be dropped in 1 cm^2 of sterile qualitative filter paper (80 g m^{-2}) forming a capsule for the gradual release of the antibiotic compounds when introduced in the culture medium. The capsules were then dried for 1 hour in laminar airflow in the absence of light and frozen under -20 ºC until their usage.

The two antibiotics, plus one control without biocides constituted the three treatments. A total of 12 sample unities were composed of 10 introduced explants, isolated in 25 mm ø essay tubes containing 10 ml of BM and a paper capsule containing ampicillin sodium salt (Figure 5), or tetracycline hydrochloride, or simply the capsule with no compound added (control). The paper capsules were replaced after 7 days. The experiment was repeated four times into a randomized complete block design (RCBD), totalizing 120 plantlets tested. After 21 days of cultivation, the frequencies of survival and contamination (fungal and bacterial) were quantified per treatment.

3.3.4 Plantlet senescence reduction and silver nitrate effects over plantlet morphogenesis and resilience

Aiming to reduce the rapid leaves senescence and the viability observed in the cultures, a preliminary test was made, adding compounds to absorb and/or restrain the action of growth inhibitors, such as phenols and ethylene. Therefore, activated charcoal (1.75 g L^{-1}) or silver nitrate – $AgNO_3$ (10 µM) was added to the basal culture medium supplemented with modified STABA vitamins (BMS) (STABA, 1969; SKIRVIN & CHU, 1979) and contrasted with a control situation without supplementation.

The three treatments were repeated five times, constituting 15 sample unities with 10 essay tubes (25 mm ø) containing 1 *in vitro* plantlet with one leaf and 10 ml of culture medium, totalizing 150 plantlets tested into an RCBD. After 30 days, the frequencies of survival, sprouts, and shoots were quantified by treatment.

Later, in order to understand the effects of different doses of $AgNO_3$ over the plants morphogenesis and estimate its optimal concentration for the cultures, the BMS were supplemented 0, 5, 15 or 45 µM of the salt. Along these, an experiment with four treatments and five replications in RCBD was carry out, containing 20 sample units with 5 essay tubes (25 mm ø) with 1 *in vitro* plantlet with one leaf inoculated in 10 ml of culture medium each,

totalizing 100 plantlets tested. The frequencies of survival, sprouts, shoots, and dead leaves were quantified in all treatments after 30 days of cultivation.

3.3.5 Different exogenous cytokines and determination of optimal concentrations in the culture medium

The evaluation of different cytokines on morphogenetic development of the cultures was performed by testing 6-Benzylaminopurine (BAP), N^6-furfuryladenine (a.k.a. kinetin, or simply, KIN), and [6-(3-hydroxybenzylamino)purine] (a.k.a. meta-topolin, henceforth, mT) at different concentrations (0, 1, 3, or 9 µM), jointly with 0.5 µM of ANA and 26 µM of $AgNO_3$ in supplementation of the BMS.

The experiment was assembled in a bifactorial design with 15 treatments, repeated five times, thus, composing 75 sample units, arranged into an RCBD. Each sample unit consisted of 5 essay tubes (25 mm ø) containing one *in vitro* plantlet with one leaf and 1 cm height, inoculated in 10 ml of culture medium.

After 30 days of cultivation, the frequency of sprouts, shoots, dead leaves, roots, and height were quantified per treatment. The morphogenetic traits were observe using a stereomicroscope (SHZ10®, Olympus™, Tokyo, Japan) and registered thought a photographic device (DP71®, Olympus™, Tokyo, Japan) paired to the equipment.

3.3.6 Statistical analysis

In order to reduce the potentiality of multiplicative effects and/or scalar distortions that inflate the variance of the data under analysis, data measured as percentages (such as survival and contamination rates) were submitted to transformation through the arcsen$\sqrt{}$ (Yi + 0.05 / 100) formula. In the same approach, counting data (such as sprouts, shoots and roots frequencies) were transformed by the log (Yi + 1) formula.

All the quantitative data collected in the experiments described above were submitted to normality and homoscedastic analysis and then, tested into an ANOVA with Scott-Knott hierarchical clustering of the means at 5% probability of error. In the cases with compounds dosage (tests with $AgNO_3$ and cytokines), the ANOVA was performed along a regression analysis inside the qualitative factor(s), in order to estimate optimal dosages to each evaluated character. All the statistical analysis were performed thought data processing in R software (R

Core Team, 2013). The regression analysis graphics were produced using the Origin Pro 8.0 software (OriginLab Corporation, Northampton, USA).

3.4 RESULTS AND DISCUSSTION

3.4.1 Ampicillin promotes efficient explant profilaxia without toxicity

Although the highest rates of survival were observed in the antibiotic-treated cultures, the differences didn't show to be significant in the statistical test performed. Notwithstanding, it demonstrates that the applied dosages were not capable of inducing observable toxicity responses in the plantlets (table 4).

Most of the contamination in *A. xanthorrhiza* cultures occurred due to associated bacteria (almost 50% of cultures, in average) contrasting with the very low fungi contamination rates (5% in average). Only one among all the sample unities presented fungi and bacteria contamination occurring together. Differently from fungi contamination, the bacterial growth in the culture medium did not predict a necessarily culture's death during the experimental time.

The ampicillin treated cultures (figure 5) showed a superior significant effectiveness to prevent and eliminate bacterial contamination (25%) against 62.5% and 60% of bacterial contaminated cultures in the tetracycline treated cultures and the negative control, respectively.

Both tetracycline and ampicillin are wide spectrum antibiotics, targeting gram-positive and gram-negative bacteria, reason why they were chosen for this experiment. Ampicillin is a β-lactam aminopenicillin that inhibits the action of the DD-transpeptidase, leaving the amino acids and sugar molecules unable to cross-link and form the peptidoglycan mesh in the bacterial cell wall. Tetracycline is a naphthacene molecule that acts by interfering in the 30S subunit of the bacterial ribosome, blocking it for tRNA binding and, therefore, stopping the transport of amino acids for peptides and proteins synthesis in the cell (GUALERZI *et al*., 2013; HAUSER, 2012; ZÄHNER & MAAS, 1972).

The rationality of using wide spectrum compounds was to find a fast, efficient, and cheap methodology, previewing situations without a proper microbiological quality assurance (LEIFERT & CASSELLS, 2001), when losses of cultures through bacterial contamination would need to be circumvented without an ideal technological support, such as biochemical identification or genotyping the contaminant by sequencing the 16S ribosomal DNA (KHAN

et al., 2018; LEONE *et al*., 2016, FHANG & HSU, 2012; LUNA *et al*., 2008), for then, decide the most indicated antibiotic treatment.

Table 4 – Averages of *A. xanthorrhiza* newly *in vitro* introduced cultures exposed to different antibiotics: survival (%), and contamination with bacteria, fungi, and total (%).

Treatment	Survival (%)**	Contamination		
		Bacteria (%)**	Fungi (%)**	Total (%)**
Control	67.50% a*	60.00% a	7.50% a	65.00% a
Ampecillin	87.50% a	25.00% b	5.00% a	30.00% b
Tetracycline	90.00% a	62.50% a	2.50% a	65.00% a
Average	*82%*	*49.17%*	*5.00%*	*53.33%*
CV%	*9.09%*	*13.24%*	*42.87%*	*14.43%*

* Means followed by the same letters in the same column do not differ according to the Scott-Knott mean separation test at 5% probability of error.

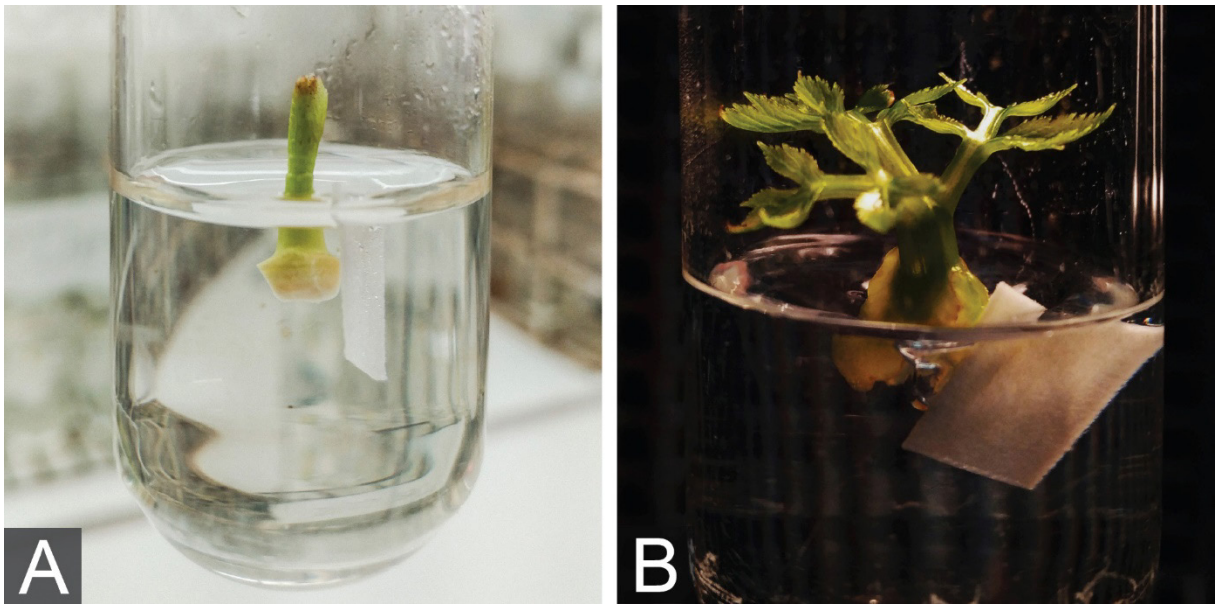
** Variable data transformed to arcsen$\sqrt{(Yi + 0{,}05/100)}$ during analysis

In the same pragmatic approach, the filter paper capsules were used to preserve the antibiotic compound, by avoiding antibiotic degradation due to the high temperatures during medium sterilization. Moreover, it is easier to preserve and store by freezing and simple to replace, supplying a new dose after plant absorption and/or the compound degradation by light, environment temperature, and oxidation, without the necessity of replacing the cultures to new vessels, therefore, saving costs with medium and handling.

Our results dialog with some reports of authors that used the same antibiotic compounds. In Khan *et al.* (2018) bacterial contaminants in *Fagonia indica* cultures (most of them, gram-positive, like *Bacillus* spp.) the treatment had a low efficacy with the use of tetracycline (16 mg L^{-1}), when compared with other shorter spectrum antibiotics, like teicoplanin and ciprofloxacin.

Although mortality and morphogenesis didn't show to differ in our data, tetracycline is long reported to induce phytotoxicity. In Palú *et al*. (2011), tetracycline showed to be highly toxic to *Ficus carica* cultures in higher dose of 500 mg L^{-1}, contrasting with a high survival and decontamination of cultures treated with an also high dose of ampicillin (250 mg L^{-1}).

Figure 5 – *A. xanthorrhiza in vitro* cultures under antibiotic treatment: A – newly introduced steam apex along with paper impregnated with ampicillin (100 mg/l); B – aseptic plantlet regenerated after 21 days of culture.



The ampicillin treatment also was reported as showing absence of toxicity effects in *Solanum tuberosum* cultures even at an inflated dose of 1.024 mg L^{-1}, opposing with tetracycline treatment, that induced toxicity even in the lower dosage tested (32 mg L^{-1}) (PEREIRA & FORTES, 2003). Using the same dosage of ampicillin that we performed our research (100 mg L^{-1}), Salehi & Khosh-Khui (1996) treated cultures of *Rosa chinensis* reaching 84% of asepsis without phytotoxicity effects (SALEHI & KHOSH-KHUI, 1996).

Aside from the foregoing, the use of antibiotic therapy is controversial in the literature. Size reduction of *in vitro* plant growth in *Ananas comosus* was reported, and deduced by the authors (LEONE *et al*., 2016), as caused by the elimination of important endophytes by antibiotic treatment. On the other hand, *in vitro* recalcitrance of *Citrullus lanatus* associated with endophytes, not manifested by bacterial growth in the culture medium, where bypassed with periodical antibiotic treatments (THOMAS, 2004).

Both examples show the difficulties into balance *in vitro* asepsis and plant inner microbiome ecology. In our case, no growth and multiplication abnormalities were verified with any of the decontaminated *A. xanthorrhiza* cultures, even when subcultivated over 24 months after the antibiotic therapy.

In view of the above, shoot tips are easier for *in vitro* introduction in industrial contexts, where not always the staff have the necessary training and qualification for meristem cultures. Therefore, the use of ampicillin emerges as a cheaper and faster strategy to obtain culture's establishment and multiplication with lower technology demand.

3.4.2 Silver nitrate *reduces in vitro* premature plantlets senescence

The preliminary essay with silver nitrate ($AgNO_3$) and activated charcoal (AC) revealed significant contrasts: meanwhile, the plants cultivated in basal medium supplemented with AC (1.75 mg L^{-1}) demonstrate lower performance than the control at all the evaluated parameters (survival rate, number of sprouts, and shoots), the plants exposed to medium with $AgNO_3$ (10 µM) exhibited substantial higher values in the three aspects compared to the other two treatments (table 5).

The Initial hypothesis was that the observed premature senescence of plants (around 21 to 30 days) was due to phenols or ethylene accumulation in the *in vitro* environment. In that scenario, the AC was an elementary candidate to work around the problem, once a vast literature in plant tissue culture reports the compound for its adsorption capacity of inhibitory molecules, such as phenols, ethylene, and hydroxymethylfurfural (THOMAS, 2008).

Table 5 – Averages of survival (%), sprouts, and shoots (n./plantlet) of *in vitro* cultures of *A. xanthorrhiza* with supplementation of different growth inhibitors suppressors in the culture medium.

Treatment	Survival (%)	Sprouts (n./plantlet)	Shoots (n./plantlet)
Control	88% b*	2.10 b	2.67 b
AgNO3 (10 µM)	98% a	3.01 a	4.62 a
Actvated charcoal (1.75 g/L)	72% c	1.23 c	1.73 c
Average	*86%*	*2.11*	*3.01*
CV%	*6%*	*0.11*	*0.14*

* Means followed by the same letters in the same column do not differ according to the Scott-Knott mean separation test at 5% probability of error.

The AC is capable to immobilize unwanted substances into the pores of its micro-crystalline structure, which presents a high specific surface area and affinity for polar organic molecules and aromatic compounds. On the other hand, the high reactivity of the AC also makes it interact with important components of the culture medium, adsorbing growth regulators and

some micronutrients, being, in some cases, deleterious to the plant morphogenetic pathway (PAN & VAN STADEN, 1998), what could indicate the most plausible cause to the cultures growth inhibition and defoliation that was observed during the essay.

Based on the preliminary results, a second experiment, dedicated to investigating an optimal concentration of $AgNO_3$, was performed: the data revealed that the compound increases survival and sprouting in all tested concentrations. Even the highest concentration tested (45 µM) was not able to overcome plant resilience to the point of lethality. However, the higher dosage was ineffective to reduce leaf senescence when compared to the experimental control (figure 6).

The AgNO3 significantly increased the number of observable shoots up to a concentration of 15 µM, decreasing when plants are exposed to 45 µM concentrations. It also reduced the number of dead leaves at 5 and 15 µM, without statistical differences between them. The concentration of 45 µM generated an increase in the number of dead leaves, equating this treatment to the experimental control, and alluding to a probable gradual toxicity of its higher levels in the culture medium.

The regression analysis determined the ideal amount to obtain the reduction of leaf senescence and increase of sprouts between 21 to 27µM, although the only strong significant correlation was found on the number of sprouts until 26 µM (97.65%).

The results found in the experiment performed over *A. xanthorrhiza* are consistent with the theoretical models and practical results obtained by other scientific papers and reviews on the subject. There is a vast literature over the usage of the AgNO3 supplementation into the culture medium as a promotor of morphogenesis in a wide range of dicots and monocots, in general, combined with cytokinins supplementation *in vitro*.

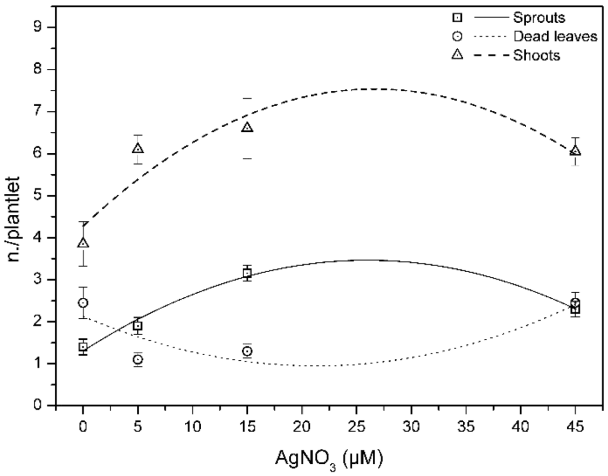
The silver ions are mainly referred to its capability of inhibit the action of ethylene – a gaseous hormone associated to the transcription and activity of stress-related genes, that mediate several metabolic pathways and morphogenetic process, promoting cells senescence, leaves and flowers abscission, fruits ripening, seed germination, and root hairs formation, whereas inhibiting lateral roots development, shoot emission, leaves growth, flowering (except in Bromeliacea), stomatal aperture and the interfering in the action of calcium signaling ions and polyamines related to organogenesis and embryogenesis (BIDDINGTON, 1992, TAIZ & ZEIGER, 2004; KUMAR, PARVATAM & RAVISHANKAR, 2009, GAO *et al*., 2017).

The $AgNO_3$ and other compounds capable of donating silver ions (like silver thiosulfate or silver nanoparticles) do not direct inhibit the ethylene synthesis and accumulation

over the whole plant but are capable of generating insensitivity of the vegetal organism to it (GEORGE, HALL & KLERK, 2008).

There is no consensus over the course of action of silver-mediated ethylene blocking, but the most accepted hypothesis is that the silver ions might replace copper ions in the bidding section of the ethylene receptor protein (ETR1), present in the endoplasmic reticulum of the cells, preventing the ethylene binding and signaling (KUMAR, PARVATAM & RAVISHANKAR, 2009).

Figure 6 – Regression analysis with estimated response, quadratic equation, coefficient of determination, ANOVA's coefficient of variation, average tendency line and confidence interval of sprouts, shoots and dead leaves (n./plantlet) of *in vitro* cultures of *A. xanthorrhiza* exposed different doses (µM) of $AgNO_3$ in the culture medium.



Variable	$AgNO_3$ (µM)	Estimated (n./plantlet)	Quadratic equation	R2	CV%
Sprouts	26	3.465	$y = -0.032x^2 + 0.1662x + 1.3065$	97.65%	10.73%*
Shoots	27	7.536	$y = -0.0046x^2 + 0.245x + 4.2741$	82.38%	6.47%*
Dead leafs	21	0.949	$y = -0.0026x^2 + 0.1108x + 2.1293$	71.31%	19.57%*

* Variable data transformed to log10 of (yi + 1) during ANOVA

Therefore, the promotion of plant regeneration and growth observed along the literature probably does not occur as a direct action of the silver ions, but as a mediation of it that allows the signaling triggered for secondary metabolites once inhibited by the ethylene (MAHENDRAN, GEETHA & VENKATACHALAM, 2019).

The *de novo* organogenesis was improved both in frequency as well as in time in the *Brassica campestris* cotyledons cultivated under 30 µM of $AgNO_3$ (CHI & PUA, 1989). Similar

results were observed in *Cucumis sativus* cotyledons and hypocotyls explants exposed to culture medium also supplemented with 30 µM of $AgNO_3$ (MOHIUDDIN, et al, 1997) and in *Manihot esculenta* under 70 µM of $AgNO_3$ supplementation, also reducing the callus formation (ZHANG, PHANSIRI & PUONTI-KAERLAS, 2001).

Giridhar *et al*. (2003) reported that *Coffea Arabica in vitro* plants exposed to 10 µM of $AgNO_3$ showed higher quantities of shoot emission, leaf area and chlorophyll content. In *Musa* spp. the *in vitro* shoot induction and rooting were improved by the addition of 59 µM of $AgNO_3$ to the culture medium (TAMIMI, 2015). *Pistacia vera* exhibited the best propagation factors, with lower callus development when cultivated in a culture medium containing 12 µM of $AgNO_3$ (OZDEN-TOKATLI, OZUDOGRU & AKCIN, 2005).

In *Capsicum frutescens*, Sharma *et* al. (2015) observed that the shoot length and the number of shoots were promoted by 30 µM of $AgNO_3$ and *in vitro* flowering was reported. Although a high differential genotypic response range, in *Solanum tuberosum*, concentrations between 5 to 10 µM of $AgNO_3$ eliminated abnormal branching and hyperhydricity in *in vitro* plantlets (TURHAN, 2004).

To sum up, due to the improvements to *in vitro* regeneration and multiplication of *A. xanthorrhiza*, the low cost, easy preparation and high solubility in water, as well as, its low phytotoxicity in the work concentration tested, it seems highly recommend supplementing *A. xanthorrhiza* cultures with AgNO3 with 26 µM in the culture medium.

3.4.3 Metapoline perform better than 6-Benzilaminopurine in *A. xanthorrhiza in vitro* organogenesis

In order to determine the optimal hormonal conditions for organogenesis in *A. xanthorrhiza*, were performed tests with different dosages (0, 1, 3 and 9 µM) of three cytokinins: 6-benzylamylopurine (BAP), kinetin (KIN) and metatopoline (mT) on *in vitro* plantlets.

A fifth concentration of 27 µM of the three growth regulators was also tested but discontinued due to the chimera formations induced in mT and BAP treatments, that showed a predominance of leaves with dramatic reduction of limb (figure 7 C), although did not predict plant mortality. Impairment of survival was not observed in any of the growth regulators under analysis, in any of the tested dosages (general average of 98.76%).

Sprouting capable of generating new plantlets (figure 7 B) and shoots emissions were significantly increased by the use of mT (figure 7 F, I and L), already at the lower dosage of 1 µM showing 3.5 sprouts and 7.2 leaves per plant, from which a concentration increase lead to little enhance at the treatment's averages but without apprehensible statistical differences between 1 to 9 µM at both variables (figure 8), demonstrating a dose-response pattern of saturation. The regression analysis inferred an optimal dosage of 4.45 µM of mT for 4.9 sprouts in average and 5.27 µM for 10.6 leaf emitted in average, but with a non-significant R^2 value to support any of them – 80.88% and 85.37%, respectively.

To obtain statistically comparable results with mT, the BAP treated plants required (figure 7 D, G and J) an addition of three times the dosage. The effects did not differ at the number of sprouts or shoots emitted after 3 µM (3 sprouts and 6.2 leaves per plant), but presented a good fitness to the regression analysis, with and R^2 of 96.6% for sprouting and 99.52% for new shoots - the interpolation the optimal dosage was calculated in 6.55 µM of BAP for 3.8 sprouts in average and 6.27 µM for 7.7 new shoots in average.

The use of KIN (figure 7, E, H and K) did not equal or overcame the effectiveness of mT or BAP in the sprout or number of leaves in any of the tested concentrations, except for the control concentration (0 µM), with low statistical fitness for sprouting (R^2 = 80.88%), only showing significance over the regression curve for shoot emissions (R = 96.56%).

KIN also showed very low biological activity in the rooting and height inhibition, expected effects of cytokinin usage (GEORGE, HALL & KLERK, 2008). Despite the large range of the confidence interval and the low fitness to the regression, the averages of plants exposed to KIN showed greater values of roots and height them those cultivated under BAP and mT, especially after at the higher dosages of 3 and 9 µM, where a statistically significant difference could be observed.

With only two exceptions focused in the use of auxins for *in vitro* establishment of apexes and seeds of *A. xanthorrhiza* (CEVALLOS FREIRE, 1991; LOPES, 2009), the investigations dedicated to its tissue culture treated the cultures with BAP as the principal growth regulator. The first report found belongs to Senna Neto (1990), who found the best shoot emissions results in *A. xanthorrhiza* using 0.44 µM of BAP, along with 0.11 µM of NAA. Later on, Luz (1993) archived the best results of shoots per plant with an increase of two-fold the BAP concentrations (0.88 µM) supplementing the PGR composition with NAA (0.54 µM) and GA_3 (0.72 µM).

Figure 7 – Morphological aspects of *A. xanthorrhiza in vitro* plantlets cultivated under different dosages 6-benzylamylopurine (BAP), kinetin (KIN) and metatopoline (mT): A – experimental control (no cytokinins supplementation); B – emerging sprout feasible of isolation to form a new *in vitro* propagule; C – limb malformation induce by 27 µM of BAP; D – BAP at 1 µM; E – KIN at 1 µM; F –mT at 1 µM; G – BAP at 3 µM; H – KIN at 3 µM; I – mT at 3 µM; J – BAP at 9 µM; K – KIN at 9 µM; L – mT at 9 µM;

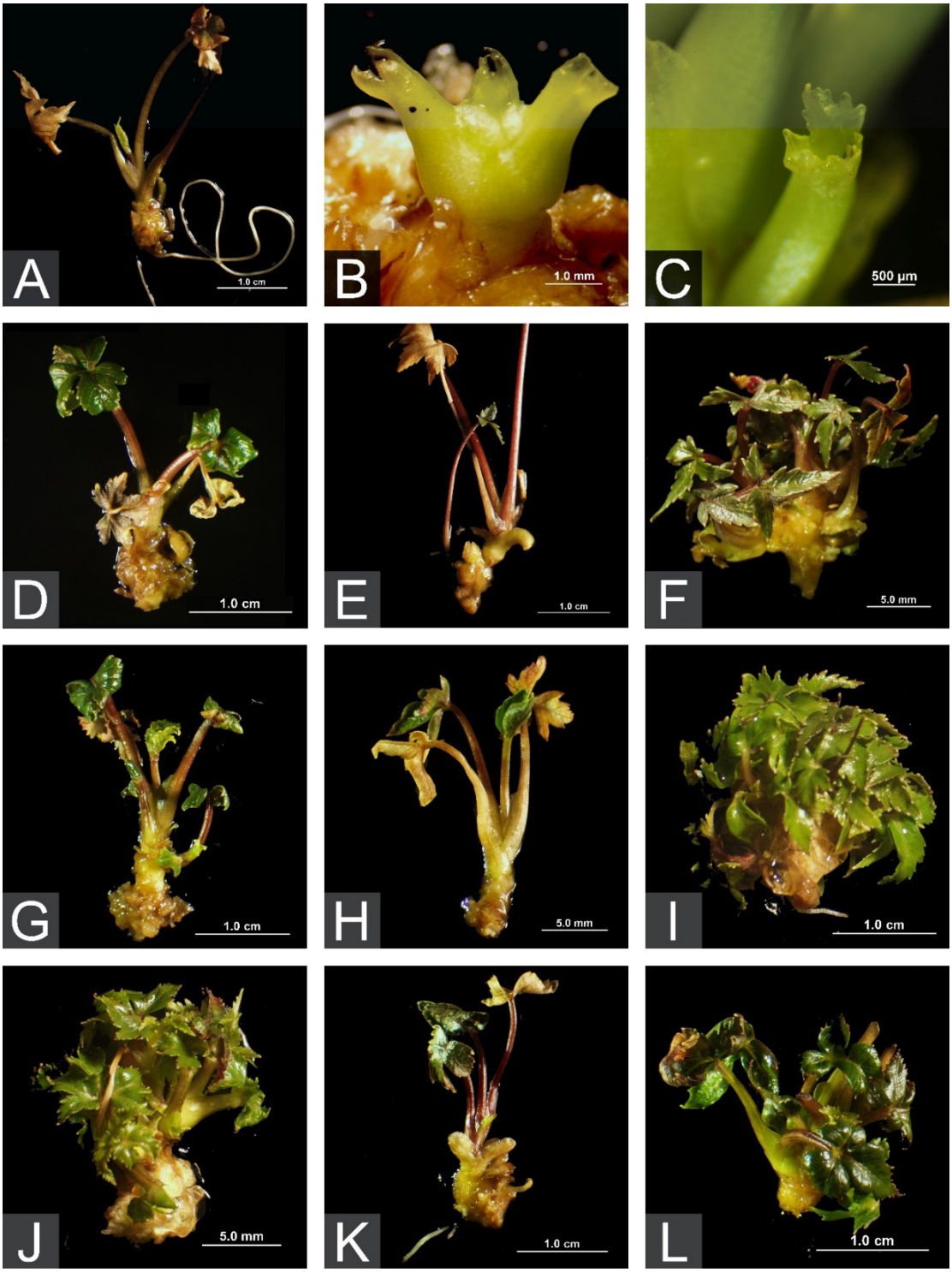
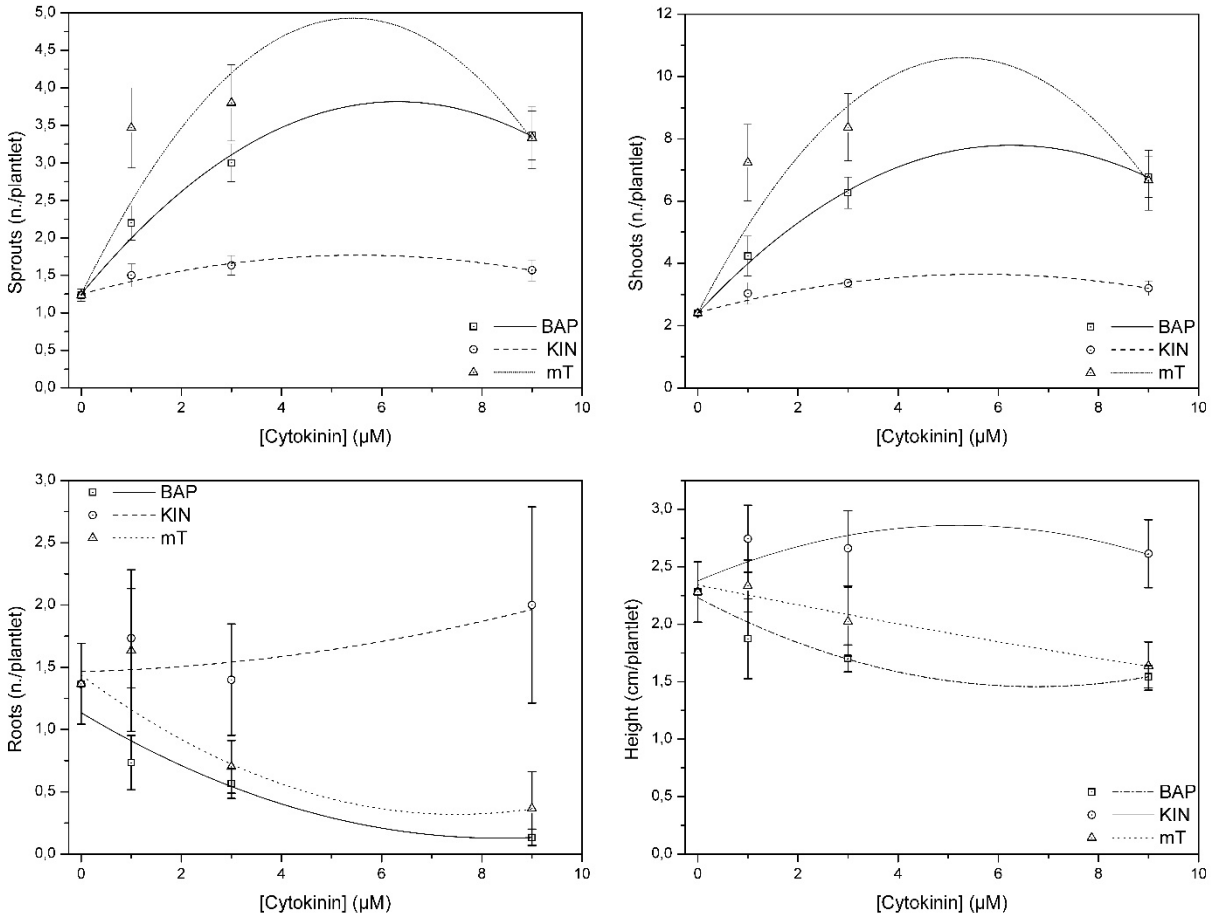


Figure 8 – Regression analysis with estimated response, quadratic equation, coefficient of determination, and ANOVA's coefficient of variation, average tendency line and confidence interval of sprouts, shoots, roots (n./plantlet) and height (cm/plantlet) of *in vitro* cultures of *A. xanthorrhiza* exposed distinct cytokines – 6-benzylaminopurine (BAP), kinetin (KIN) and meta-topolin (mT) – different doses (µM) in the culture medium.



Variable	Cytokinin	[Optimum] (µM)	Estimated response	Unit	Quadratic equation	R2	CV%
Sprouts	BAP	6.55	3.813	(n./plantlet)	$y = -0.0644x^2 + 0.81338x + 1.24786$	96.60%	15.77%*
	KIN	5.91	1.767		$y = -0.01708x^2 + 0.18896x + 1.24663$	88.63%	
	mT	5.45	4.930		$y = -0.12574x^2 + 1.36106x + 1.24653$	80.88%	
Shoots	BAP	6.27	7.794	(n./plantlet)	$y = -0.13774x^2 + 1.72341x + 2.40377$	99.52%	12.97%*
	KIN	5.64	3.648		$y = -0.0393x^2 + 0.44092x + 2.4115$	96.56%	
	mT	5.27	10.603		$y = -0.29136x^2 + 3.09033x + 2.40842$	85.37%	
Roots	BAP	0.00	1.135	(n./plantlet)	$y = 0.01436x^2 - 0.24041x + 1.13482$	86.09%	57.17%*
	KIN	9.00	1.963		$y = 0.00502x^2 + 0.0101x + 1.46557$	87.67%	
	mT	0.00	1.437		$y = 0.01981x^2 - 0.29765x + 1.43704$	74.92%	
Height	BAP	0.00	2.231	(cm/plantlet)	$y = 0.01699x^2 - 0.2293x + 2.231$	91.53%	20.65%*
	KIN	5.18	2.860		$y = -0.01775x^2 + 0.18526x + 2.37617$	30.78%	
	mT	0.00	2.343		$y = 0.0012x^2 - 0.08952x + 2.34259$	89.34%	

* Variable data transformed to log10 of (yi + 1) during ANOVA

Those first reports were centered to establish a regeneration protocol from meristems, in order to guarantee pathogen free plants. Duque & Landázuri (1999), Madeira (2004), Slíva, Viehmannová & Vítámvás (2010), and Matos *et al*. (2015) seemed, overall, focused into develop a viable *in vitro* protocol for multiplication, although most of them measured the number of plants leaves and roots, height and size of what was called as "*basal callus*". The only one that evaluated the number of sprouts, where the potential for cutting and multiplication is formed were Duque & Landázuri.

Madeira (2004) and Matos *et al.* (2015), using Brazilian and Venezuelan genotypes, respectively, reached the same conclusions about the most adequate concentration of BAP at 1.33 μM, supplemented with 0.54 μM of NAA, in order to induce a pattern of morphogenesis with low basal callus and maximum shoot levels per plant. Madeira also used GA_3 (0.72 μM) in the culture medium, looking for an alleviation of the dwarf pattern in plants cultured under BAP.

At Slíva, Viehmannová & Vítámvás (2010) experiments, the *A. xanthorrhiza* cultures reached the maximum regeneration and development in culture medium supplemented with BAP a 4.44 μM and ANA a 0.54 μM. The study of Duque & Landázuri (1999) is an interesting point out of the curve, were the higher sprouting with regular development of the plants was obtained under 24.86 μM of BAP. Their experiments only found abnormalities in the regenerated organs under a massive dose of 35.62 μM of BAP, when the plants started to present development inhibition and limb deformities, the same description of the effects that we verified using 27 μM of BAP.

The results that are presented in experiments performed in the present work reveal important points of consonance and deviation with some observations made in those previous works about cytokinin levels and its effects over *A. xanthorrhiza in vitro* morphogenesis (table 6).

Granting that, in a first effort, a curve of cytokinin concentrations was developed trying to interlope all the previous results in the literature – covering from 0 to 27 μM – is remarkable that very toxic levels of BAP and mT were encountered at the higher dosage, while Duque & Landázuri only came across the problem of the limb deformations using 25% more cytokinin concentration, even though the mentioned authors worked with very distinct Peruvian genotypes.

Table 6 – Use of saline formulations, growth regulators and work concentrations reported as the best combinations for the establishment and multiplication of organogenic cultures compared to the presented results.

Report	Saline Formulation	Growth Regulators and Work Concentration (µM)				
		NAA	IAA	BAP	mT	GA3
Sena Neto (1990)	B5	0,11	-	0,44	-	-
Luz (1993)	B5	0,57	-	0,88	-	0,72
Cevallos Freire (1991)	MS	-	0,29	-	-	0,72
Duque & Landázuri (1999)	MS	0,27	-	24,86	-	-
Madeira (2004)	B5	0,54	-	1,33	-	0,72
Slíva *et. al* (2010)	MS	0,54	-	4,44	-	-
Matos *et. al* (2015)	B5	0,54	-	1,33	-	-
Presented results	**B5**	**0,50**	**-**	**-**	**1,00**	**-**

Madeira (2004) encountered limitations to propagation after 1.33 µM of BAP using the same cultivar tested in the data shown in this work, and used the basal medium only differed in the vitamin's composition and the addition of $AgNO_3$, leading to two possible and not excluding interpretations: there is a positive interaction in the medium salt's supplementation proposed or/and there are a possible epigenetic or even genetic differences between the accession used.

The major difference to detail from the past works is the multiplicative performance of *A. xanthorrhiza* plants treated with mT to induce the same number of sprouts and shoots with a third of the BAP dosage. The mT and the BAP are aromatic cytokinins, but mT differs in the presence of a hydroxyl group bounded at the benzyl ring, which is credited as the main factor for its higher biological activity *in planta* (SAKAKIBARA, 2006; SUBBARAJ, 2011).

When the first major investigations on the specie were developed, between the decades of 1990 to 2000, the topolins were yet restricted to specialized biochemical studies. Although the identification of the hydroxylated analogues of 6-benzylaminopurine happened in the decade of 1970, only with the technologies available around twenty years later, the topolins biosynthesis and activity began to be comprehend (SUBBARAJ, 2011).

At the present time, the topolins are still costly, compared to other aromatic and isoprenoid cytokinins, but are in increasing usage in the research and production areas of plant tissue culture due to its broad recognizing potential for augmenting plant multiplication and alleviating some problems caused by the traditional cytokinins, mainly BAP, such as chimera formations, hyperhydricity, histogenetic instability, phytotoxicity, rooting, and height

inhibition (AREMU *et al*. 2012; AMMO, FINNIE & VAN STADEN, 2011; DOBRÁNSZKI *et al*., 2005).

Those advantages cannot be generalized to the *A. xanthorrhiza* micropropagation under mT stimulation, once the malformations rooting and height inhibition were verified in a very similar pattern with BAP after 3 µM, although mT showed slightly higher averages and larger confidence interval of response. The main benefit verified is at the propagation's factors archived by very low dosages, that potential bring more safety of genetic stability and reduced costs for regenerated clones.

The plants cultivated in mT dosage of 1 µM were subsequently transferred to the rooting medium with the same basal medium composition, without cytokinins, supplied with 3 µM of NAA and 0.7 µM of GA_3, showing 78% of rooted plants and an average of 3.4 roots per plant, with no observable abnormalities, after 30 days of cultivation.

3.5 CONCLUSIONS AND FUTURE PROSPECTS

The proposed improvements circumvent or overcame some typical problems of plant tissue culture that are also encountered in the *A. xanthorrhiza* micropropagation. The ampicillin supplementation at 100 mg L^{-1} dosage allowed an effective and low-cost method for bacterial contaminants elimination and culture establishment. The use of AgNO3 at the dosage of 26 µM promoted new sprouts and shoots along with a decrease in the plant's senescence, enhancing the multiplication factors and allowing more plasticity in the period to subcultive the plants. The use of mT at 1µM saturates the dose-response of the plants for sprouts and shoot emission, showing three-fold more biological activity and lower inhibition effects for roots emission than the commonly used BAP.

There are still many knowledge gaps about the genetic fidelity and the ability to embrace the genetic diversity existing in the species of the organogenesis protocol proposed. In this matter, new tests with landraces and breeding lines are a demand for the future, as so as flow cytometry and molecular genetic analysis of the regenerated plants, looking for an ensured high yield and true-to-type *in vitro* regeneration protocol.

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4 CAPÍTULO II – *IN VITRO* MORPHOGENESIS OF *ARRACACIA XANTHORRHIZA* BANCR. ("ARRACACHA") PLANTLETS CULTIVATED IN DIFERENT GAS EXCHANGE INTENSITIES AND TEMPORARY IMMERTION BIORREACTORS

4.1 ABSTRACT

The use of bioreactors in plant tissue culture is largely reported as an effective way to scale up production and promote plants' physiological and morphological qualities, meanwhile reducing handling, production time, and acclimatization losses. Therefore, temporary immersion bioreactor systems (TI) emerged in commercial laboratories as the main way to reduce production costs toward economic viability. Here we present the first reported experiments aiming at understanding the morphogenetic features of *Arracacia xanthorrhiza* plants cultured under passive gas exchange (GE) and TI systems in a comparative way to the classic in agar and hermetic propagation environments. Plantlet development was evaluated under different GE intensities (0, 9.87, 13.09, 15.58, and 81.35 GE/day) and comparisons were performed between semisolid and liquid medium over vermiculite substrate, both with and without GE, as so as in TI bioreactors RITA® and twin flasks. The stomata densities, morphology, and behavior were also observed through diaphanization and electron scanning microscopy. The GE and TI plants had no significant differences between semisolid and hermetic culture environments for sprouts and shoots emissions. Rooting was only induced under vermiculite presence, and hyperhydricity occurred only on TI plants (8.33%), which had a significant higher mass per plant, but the dry mass content was statistically equal in all treatments, indicating that GE and TI do not promote an autotrophic behavior. Nonetheless, GE and TI led to a higher frequency of normal morphology and functional stomata, than those observed in hermetic culture conditions, that had predominance of opened, elevated and circular shape stomata.

Keywords: Apiaceae, Peruvian carrot, SEM, autotrophy, stomata.

4.2 INTRODUCTION

The arracacha (*Arracacia xanthorrhiza* Bancr.) is an Andean tuberous root cultivated in the in humid highlands of South and Central America countries, representing part of the

dietary of 80 to 100 million people in those regions (HERMANN, 1997), and an important source of income for family farmers and local markets.

It is the only Apiaceae domesticated in the Americas through asexual propagation (MORILLO & SÉCOND, 2017), that consists in the isolation and rooting of cormels – a technique that is being carried out to the present days and contributed to the dissemination of crop and soil diseases, leading to the economic losses and genetic erosion of the cultivated forms (HENZ, 2002; MADEIRA, 2004).

Despite the effort to develop micropropagation protocols that support *in vitro* clonal gardens to supply the demand for healthy clones for the conservancy, breeding, and crop fields, none of the proposed methods had, so far, an efficacy to a massive scale up the propagules and reducing handling costs to ensure an economically viable way for industrial micropropagation laboratories, especially due to the classic approach on *in agar* propagation verified along the erstwhile investigations.

The use of bioreactors in plant tissue culture emerged in the horticulture industry as an effective way to reduce expenditures with handling for shearing plants and time for production inside the lab, avoiding the use of expensive and labor costly gelling compounds and losses in the acclimatization phase, meanwhile, promoted the automation of the process and enhanced the propagation factors with gains in propagules physiological and morphological qualities in several species, steering estimated reductions of 40 to 60% in the production costs (ETIENNE & BERTHOULY, 2002; GEORGIEV et al., 2014).

In that context, a comprehension of the *in vitro* behavior of *A. xanthorrhiza* morphogenesis in bioreactors systems represents the biotechnological base through an economic feasible way to supply farmers, breeders, conservationists, and plant science researchers with healthy and genetic quality propagules.

Thus, the investigations presented in this paper were driven to understand the effects of gas exchanges in *A. xanthorrhiza* morphogenesis and comparing its micropropagation performance and morpho-physiological traits through cultivation in classic *in agar* culture with gas exchange, liquid media environments, and temporary immersion bioreactor systems.

4.3 MATERIAL AND METHODS

4.3.1 Plant material

All tests were performed with *in vitro* plants previously established (through six prior subcultives) from mother plants kept in greenhouse conditions. The matrix plants originated from cormels of healthy and vigorous plants selected from production in the fields of family farmers, in the municipality of Angelina (S 27.456.258, E 49.063.053), South region of Brazil.

The cultivar *Amarela de Senador Amaral* (ASA) was selected to execute the tests that compose the basics data to support the protocol development, due to its representativeness in the crop fields, occupying over of the cultivated areas in the country.

4.3.2 Evaluation of gas exchange, liquid and semisolid medium over plants morphogenesis

In order to evaluate the effects and optimal gas exchange (GE) of the plants' headset per day in static medium conditions, a pilot test was performed, comparing five different situations: 0, 9.87, 13.09, 15.58, and 81.35 GE/day, using 80 mm ø and 40 mm height O95/40+OD95 Microbox® micropropagation containers and hermetic covers or with different mesh GE filters from SACO2™ (Deinze, Belgium). The 5 treatments (GE/day) were repeated 5 times, composing 25 sample units, arranged into a RCBD. Each sample unit consisted of 1 Microbox ø) containing 5 *in vitro* plantlets with 1 cm height, one leaf, and filled with 30 ml of culture medium.

In a further experiment, we performed a comparison between the morphogenetic characters of plants grown in static liquid and semisolid medium (with and without GE) along with plants in different temporary immersion bioreactors systems (TI).

The plants in static medium were cultivated in 90 mm ø and 140 mm height O119/140+OD119/140 Microbox® micropropagation containers with a hermetic cover or a cover with a filter for 15.58 GE/day. The semisolid medium was gelified with Phytagel™ 0.2% (w./v.) and the liquid medium was embedded in 100 ml of vermiculite substrate in order to sustain the plants' position and aeration. The plants in bioreactors were cultivated in temporary immersion RITA® (VITROPIC™, Saint-Mathieu-de-Tréviers, France) (henceforth, TI-RITA) or in temporary immersion twin-flasks (TI-TF) assembled with two 500 ml glass erlenmayers, closed with silicone stoppers, drilled with glass ferrules along with silicone hoses for connection of plants and medium vessels, enabling air and medium flow in the system. In both bioreactors, the air was sterilized thought 1 bar pressure input over polypropylene hydrophobic filtering membranes with 0.45 pores sizes (Merck Millipore Millex™, Burlington, USA).

All the vessels and bioreactors received 15 *in vitro* plantlets with 1 cm height and one leaf, and were filled with 200 ml of culture medium. A total of 6 treatments were repeated 5 times, composing 30 sample units, arranged into a RCBD.

In both experiments, after 30 days of cultivation, the frequency of survival, sprouts, shoots, defoliation, fresh mass, dry mass, dry matter, and water content were quantified per treatment, and the morphogenetic traits were observe using a stereomicroscope (SHZ10®, Olympus™, Tokyo, Japan) and registered thought a photographic device (DP71®, Olympus™, Tokyo, Japan) paired to the equipment and a DSLR digital camera (EOS Rebel T3i® with Lens EF-S 18-135mm 1:3.5-5.6 F IS®, Canon™, Tokyo, Japan).

4.3.3 Culture medium and cultivation conditions

The plants were cultivated in medium based on B5 saline formulation (GAMBORG, MILLER & OJIMA, 1968), supplemented with modified STABA vitamins (STABA, 1969; SKIRVIN & CHU, 1979), 3% sucrose (w./v.), 0.5 µM of α-Naphthaleneacetic acid (NAA), 1 µM of meta-topolin (mT), and 26 µM of $AgNO_3$, with pH adjusted to 5.8 with 1N KOH, before sterilization in autoclave at 121 °C for 15 min.

The vessels and bioreactor were incubated in growth room at 25 ± 2 °C, 60% ± 10% of relative humidity and a photoperiod of 16 hours under 50 µM m^{-2} s^{-1}, supplied by white spectrum LED lamps (GreenPower TLED W®, Philips™, USA). Each sample unit, was composed with one vessel or bioreactor and counted with 15 *in vitro* plantlets. The bioreactors were supplied with 1 bar of compressed air for 3 min. with 3 hours cycle intervals.

4.3.4 Statistical analysis

In order to reduce the potentiality of multiplicative effects and/or scalar distortions that inflate the variance of the data under analysis, information measured as percentages (such as survival and density rates) were submitted to transformation through the arcsen$\sqrt{}$ (Yi + 0.05 / 100) formula. In the same approach, counting data (such as sprouts, shoots, and roots frequencies) were transformed by the log (Yi + 1) formula.

All the quantitative data collected in the experiments described above were submitted to normality and homoscedastic analysis and then, tested into an ANOVA with Scott-Knott

hierarchical clustering of the means at 5% probability of error. All the statistical analysis were performed thought data processing in R software (R Core Team, 2013).

4.3.5 Diaphanization and scanning electron microscopy for stomatal counting and morphology evaluation

Stomata counting and morphology observations were made from fully expanded leaves of *in vitro* plants. To achieve so, the tissues were diaphanized, following Kraus & Arduin (1997) recommendations, with specific adaptations due to the sensitivity of the material. The leaves were excised and fixed in a buffer of ethanol (63% v./v.), acetic acid (18.5% v./v.), and formaldehyde (18.5% v./v.) for 48 hours, under vacuum condition, followed by immersion in NaOH 5% (w./v.) for 30 min, immersion in NaOCl 2,5% (w./v.) for 2 hours and dehydrate under ethanol by series of 50%, 70% 90% and 100% for 30 min. each.

To clarify the tissues, the leaves were immersed in an ethanol-xylene (1:1) for 30 seconds, followed by xylol for more 30 seconds, rinsed in deionized water, and colorized by two series of 1% toluidine blue (w./v.) and 1% sodium tetraborate (p./v.) and finally rinsed with deionized water of 3 minutes. The leaves were mounted into slides with cover glass, closed with DPX Mountant for histology (Sigma-Aldrich Corporation™, USA).

The material was observed under an optical microscope (Olympus BX-40®, Olympus Center Valley, USA) and photographed (Olympus DP71®, Olympus Center Valley™, USA). For each treatment in evaluation, three random optical fields along the limb were photographed and analyzed with the software ImageJ® (LOCI™, University of Wisconsin; COLLINS, 2007). The ANOVA statistical analysis of the stomata distribution was performed through a data process in R software (R Core Team, 2013).

For the analysis of the stomata morphology and distribution by scanning electron microscopy (SEM), fully expanded leaves of *in vitro* plants were fixed in 2.5% paraformaldehyde solution (p./v.) and 0.1 M sodium phosphate buffer (pH 7.2) for 24 hours, under vacuum condition, and then washed in sodium phosphate buffer 0, 1 M (pH 7.2) and dehydrated in an ethanol series (from 20 to 100%), with a 30 min. period at each alcoholic graduation, following the methodology of Ruzin (1999).

The samples were then dehydrated according to Horridge & Tamm (1969) protocol, using a CPD 030 Critical Point Dryer (Leica™, Heilderberg Germany), stick on aluminum supports with double-sided carbon tape, and covered with 30 nm film of gold in a SCD500 high

vacuum film deposition system (Leica™, Heilderberg Germany). The images were captured in a JEOL XL30® scanning electron microscope (JEOL™, Tokyo, Japan) at 10kV and amplification from 100 to 3000x. The full process was performed at the Laboratório Central de Microscopia Eletrônica (LCME) of the Universidade Federal de Santa Catarina (UFSC), in Florianópolis, Brazil.

4.4 RESULTS AND DISCUSSTION

4.4.1 Gas exchange intensity plays minor role in *in vitro* survival of the plantlets

The use of GE membranes did not affect the survival rates of the plants. Along all the GE treatments in the pilot experiment, the average survival was 96.80% and the defoliation average was 1.3 dead leaves per plant, with no significant differences between the treatments in both cases (table 7 and figure 9).

Table 7 - Means of survival (%), sprouts, shoots, dead leaves, roots (n. per sample unity), hyperhydricity (%), fresh mass, dry mass (mg/plant) dry matter and water content (%), of *in vitro* cultures of *A. xanthorrhiza* plantlets cultivated in under different gas exchange (GE) intensities per day

		Survival (%)	Sprouts (n./plantlet)**	Shoots (n./plantlet)**	Dead leaves (n./plantlet)**
G.E./day	**0**	98,00% a*	1,88 a	3,86 b	1,20 a
	9,87	96,00% a	1,92 a	4,30 b	1,62 a
	13,09	94,00% a	1,72 a	3,92 b	1,30 a
	15,58	100,00% a	2,14 a	5,16 a	1,20 a
	81,35	96,00% a	1,46 b	2,90 c	1,18 a
	Average	*96,80%*	*1,82*	*4,03*	*1,30*
	CV%	*7,76%*	*14,92%*	*13,15%*	*20,00%*

		Fresh mass (mg/plantlet)	Dry mass (mg/plantlet)	Dry matter (%)***	Water content (%)***
G.E./day	**0**	103,84 a	12,92 a	12,14% b	87,86% a
	9,87	141,43 a	18,90 a	12,98% b	87,02% a
	13,09	140,59 a	20,52 a	14,45% b	85,55% a
	15,58	136,51 a	21,01 a	15,36% b	84,64% a
	81,35	56,69 b	13,88 a	25,08% a	74,92% b
	Average	*115,81*	*17,45*	*16,00%*	*84,00%*
	CV%	*37,56%*	*39,28%*	*8,25%*	*4,05%*

* Means followed by the same letters in the same column do not differ according to the Scott-Knott mean separation test at 5% probability of error.

** Variable data transformed to log10 of (yi + 1) during analysis.

** Variable data transformed to arcosene [√ (yi + 0.05) / 100] during analysis

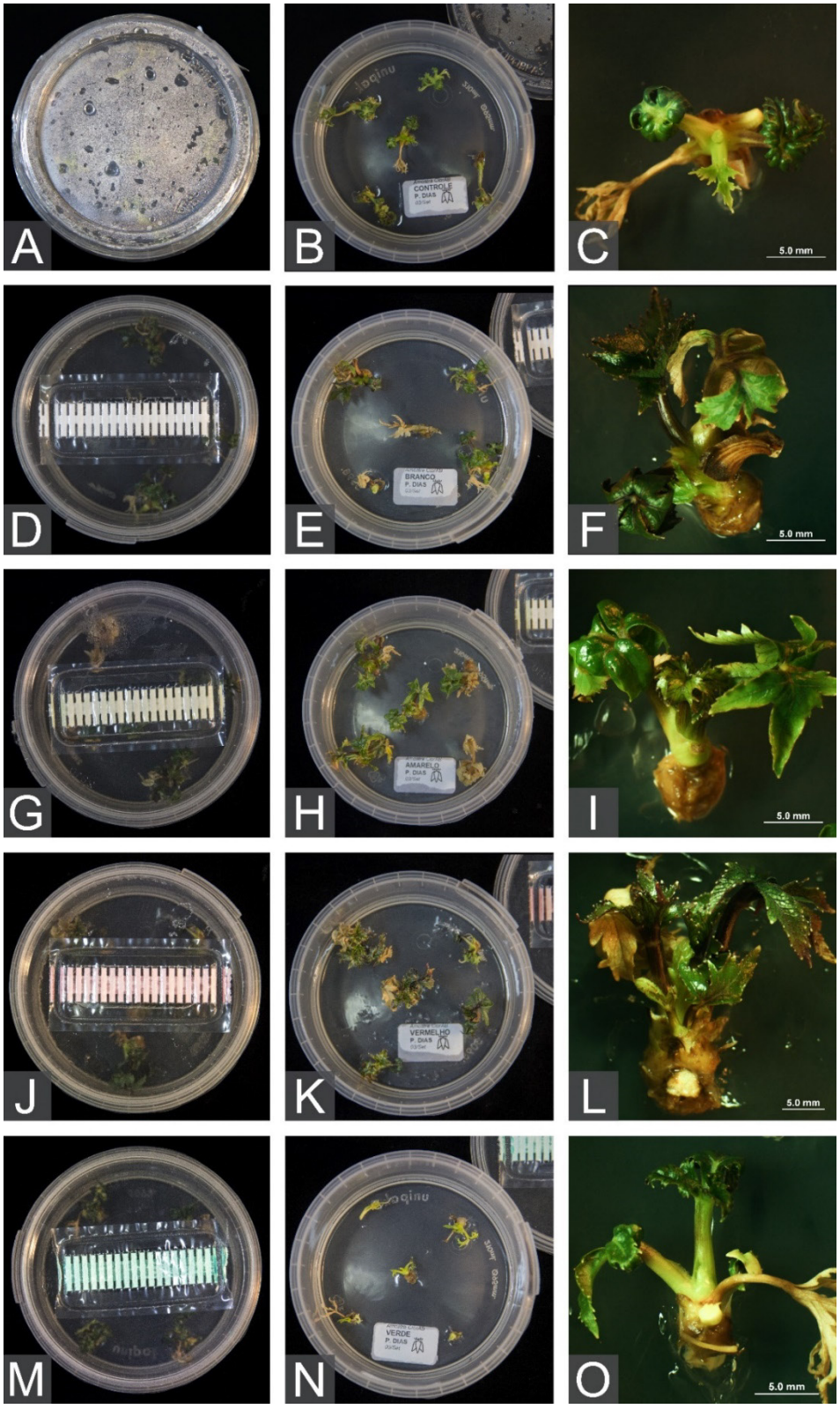
It was expected a raise of sprouts and new leaves emission averages in GE to gradually occur as a result of ethylene elimination (BIDDINGTON, 1992, KUMAR, PARVATAM & RAVISHANKAR, 2009, GAO et al., 2017) and raise of CO_2 levels and photosynthetic activity by the augmentation of air flux (KITAYA *et al*. 2005; KOZAI, 2010) athwart the membranes. Nevertheless, the significant variation appeared only at the increase of the GE to 81.35 GE/day, leading to a severe reduction of sprouts average (1.46 sprouts per plant), meanwhile all the other treatments demonstrated to be statically equal.

The higher GE/day rate also had the lower shoots per plant (2.9) that in any other treatment. Plants that were cultivated under 15.58 GE/day showed the best performance (5.16 shoots per plant), but the use of hermetic covers and GE/day of 9.87 and 13.09 remained in an intermediate level, with no significant differences between them.

Although the demonstrated best results for sprouts and shoot emissions with membranes that allowed 15.58 GE/day and no sucrose reduction in the medium composition was made, the dry mass showed that the GE was unable to enhance the carbon fixation and scape from a mixotrophic towards an autotrophic regime (DESJARDINS, HDIDER & DE RIEK. 1995), as expected, if not, as a dehydration symptom in the higher GE rate in a deleterious condition for plants morphogenesis, as just mentioned.

The fresh and dry matter and water content only showed significant differences in the treatment with the highest GE/day, probably due to severe dehydration in the plants, that the presented lower values in all the evaluated sample unities, demonstrating that, such higher exposures to GE should be used only for short periods before acclimatization or gradually introduced to the cultures, both hypothesis worth to be future tested.

Figure 9 - General aspects of *A. xanthorrhiza* plantlets morphogenesis under different gas exchange (GE) intensities: A, B and C – hermitic cover; D, E and F – 9.87 GE/day; G, H and I – 13.09 GE/day; J, K and L – 15.58 GE/day; M, N and O – 81.35 GE/day;



4.4.2 GE and TI enhance *in vitro* survival, growth, and sprouting, but TI generates plantlets hyperhydricity

In order to compare classic *in agar* micropropagation with passive gas exchange systems, liquid media environments and temporary immersion bioreactor systems, a series of preliminary tests were made in order to play the fairest comparisons of such different situations. The results of the aforementioned experiment were the criteria to the use of 15.58 GE/day in passive GE conditions.

To compare static liquid medium with the other culture situations, a trial test was performed with 200 ml of medium, the minimum volume required in order to operate TI-RITA and TI-TF bioreactors. In such volume of liquid, the plants, naturally, got submersed by the medium, dying for anoxia and hyperhydricity effects in a 10-day period.

A circumvent for the problem was searched in the nutrient of inert substrates to embed with medium. Therefore, tests were made with 100 ml of perlite (volcanic glass mineral) and vermiculite (phyllosilicate mineral), in order to sustain the plants and enable proper aeration. As the first one simply floated with no aggregation, the choice for vermiculite substrate was made in the tests with liquid medium, generating unexpected contrasts, as discussed frontward.

The general rate of plant survival to the treatments was of 97.11% average, with no statistical differences between them. Notwithstanding, the adaption and development of the plants to the different conditions showed some remarkable contrasts among liquid medium with vermiculite and the semisolid and TI conditions (table 8 and figure 10).

The sprouting showed higher averages at the plants cultivated in TI-RITA (3.3 sprouts/plant), semisolid medium with gas exchange (SS-GE) (3.3 sprouts/plant), IT-TF (2.78 sprouts/plant) and semisolid medium hermetic (2.7 sprouts/plant), with no differences among them. The treatments with liquid medium over vermiculite with gas exchange (LQ-GE) and hermetic (LQ-H) presented the lower values, with no significant variations between them (1.70 and 1.78 sprouts/plant, respectively).

The same tendency was observed on the number of shoots per plant, being statistically superior in TI-RITA (9.33), SS-GE (7.77), TI-TF (6.83), and SS-H (6.28), with no differences among the treatments and demonstrating lower emission on LQ-H with (3.68) and LQ-GE (4.05). The defoliation measured by the number of dead leaves followed an inverse pattern, presenting higher mean values on both treatments with liquid medium and vermiculite.

Table 8 - Means of survival (%), sprouts, shoots, dead leaves, roots (n. per sample unity), hyperhydricity (%), fresh mass, dry mass (mg/plant) dry matter and water content (%), of *in vitro* cultures of *A. xanthorrhiza* plantlets cultivated in differential medium availability (semisolid and liquid) and different gas exchange systems (hermetic, passive and active gas exchange)

	Survival (%)**	Sprouts (n./plantlet)***	Shoots (n./plantlet)***	Dead leaves (n./plantlet)***	Roots (n./plantlet)***
Ss hermetic	93,33% a*	2,70 a	6,28 a	2,20 a	0,00 c
Lq hermetic	91,67% a	1,70 b	3,68 b	1,48 b	8,50 b
Ss gas exchange	91,67% a	3,30 a	7,77 a	2,67 a	0,00 c
Lq gas exchance	90,00% a	1,78 b	4,05 b	1,83 a	16,25 a
TI Twins Flasks	88,33% a	2,78 a	6,83 a	1,15 b	0,25 c
TI RITA	91,67% a	3,30 a	9,33 a	1,15 b	0,00 c
Average	*91,11%*	*2,59*	*6,33*	*1,75*	*4,17*
CV%	*30,30%*	*12,32%*	*10,12%*	*19,46%*	*46,01%*

	Hyperhydricity (%)**	Fresh mass (mg/plantlet)	Dry mass (mg/plantlet)	Dry matter (%)**	Water content (%)**
Ss hermetic	0,00% a	206,65 c	17,73 c	8,69% a	91,31% a
Lq hermetic	0,00% a	197,62 c	16,70 c	8,78% a	91,22% a
Ss gas exchange	0,00% a	246,08 c	21,53 c	8,73% a	91,27% a
Lq gas exchance	0,00% a	148,98 c	13,47 c	9,05% a	90,95% a
TI Twins Flasks	8,33% b	558,08 a	54,13 a	9,64% a	90,36% a
TI RITA	8,33% b	423,72 b	42,77 b	10,07% a	89,93% a
Average	*2,78%*	*296,86*	*27,72*	*9,16%*	*90,84%*
CV%	*90,35%*	*18,76%*	*22,32%*	*3,45%*	*1,73%*

* Means followed by the same letters in each of the analyzed variables do not differ according to the Scott-Knott mean separation test at 5% probability of error.

** Variable data transformed to arcosene [√ (yi + 0.05) / 100] during analysis

*** Variable data transformed to log10 of (yi + 1) during analysis

Although the poor performance on shoots and sprouts development, the LQ-H and LQ-GE treatments demonstrated to be the only permissive conditions for root emissions, significantly enhanced by the gas exchange (16.25 roots per plant). With the exception of the TI-TF, which had only 0.25 roots per plant in average, no roots were observed in the other treatments.

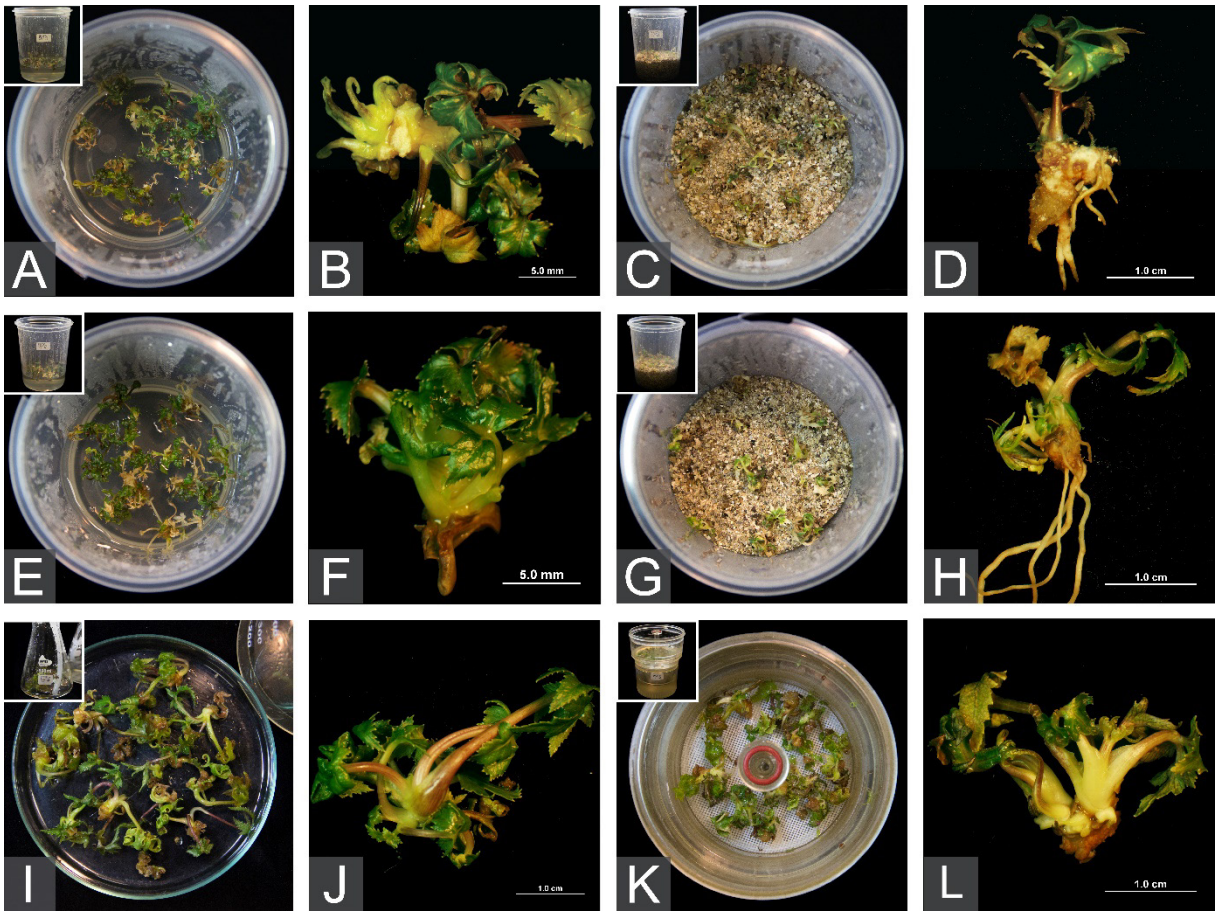
The vermiculite does not add nutrients to the culture environment, but, due to its enhanced cation exchange capability, especially with divalent cations, important medium macronutrients like Mg and Ca, and micronutrients as Co and Cu can be strongly adsorbed by the phyllosilicate surfaces until a solution equilibrium is reached (WILD & KEAY, 1964; FONCESA *et al*., 2005).

Owing to the clear sprouts and shoots development difficulties of the plants cultivated in vermiculite, it is reasonable to assume that those are may occur due to nutrient depletion, in

a similar way as verified when *A xanthorrhiza* plants are exposed to activated charcoal in the culture medium (chapter II, item 3.4.3).

Anyhow, the nutrients reduction seems to promote the rooting of the plants. The behavior in the growth regulators in a vermiculite substrate is unknown, but a charge mediated restriction of cytokinin might be a feasible hypothesis, as so as, the use of such environment for final rooting of TI scaled up plants, along with GE, in a complementary way, is a test worth to be made (VIDAL & SÁNCHEZ, 2019).

Figure 10 – General aspects of *A. xanthorrhiza* plantlets morphogenesis under differential medium availability (gelled and liquid) and gas exchange systems (hermetic, passive and active gas exchange): A & B – gelled medium with no gas exchange; C & D – liquid medium and vermiculite with no gas exchange; E & F – gelled medium with passive exchange; figures G & H – liquid medium and vermiculite with passive exchange; figures I & J – temporary immersion bioreactor with twin flasks; figures K & L – RITA temporary immersion bioreactor;



Hyperhydricity was only noticed on the bioreactors, with no differences between them (8.33%), perceptible on the petioles and limbs of the leaves, along with prominent hypertrophy deformations. TI-TF plants presented the highest fresh mass (558.08) followed by TI-RITA plants (432.72). The other treatments showed no significant differences in this measure. Measuring the dry matter/water content, all the treatments showed no significant differences between them, meaning, as in the GE experiments, no enhancement of autotrophy and carbon fixation by the plants.

After the experimental time, the bioreactors plants (TI-RITA and TI-TF) were submitted to a medium exchange, in an attempt to induce root formation. The salts and vitamins of the medium composition were kept, but the cytokinin was removed and supplementation with 3 µM of NAA and 0.7 µM of GA_3 was made. In 20 days almost 90% of the plants showed severe hyperhydration and oxidation, with no root emissions and posterior death, a very contrasting situation when the same procedure is made successfully with the classic *in agar* cultivated plants (chapter II, item 3.4.4).

Due to the higher mass of the TI-grown plants, with no increase of the dry mass, is possible that the hyperhydration effects were beyond the observable at naked eye, demanding future histological analysis. The reduction of medium immersion time, larger intervals of medium supply, and increase of the aeration without medium irrigation is a current technique to avoid the hyperhydration problem (CARVALHO *et al*., 2019; VIDAL & SÁNCHEZ, 2019) and might as well promote the photoautotrophic condition.

4.4.3 GE and TI induce morpho-physiological adaptations for *ex vitro* development of the plantlets

The stomata density and functionality along the environmental condition are crucial morpho-physiological traits of micropropagated plants' adaptability to *ex vitro* conditions. In turn, passive (thought membranes) and mostly, active (in bioreactors) gas exchange are, in general, the way to achieve it due to the elimination of humidity and rise of the CO_2 levels in the vessel atmosphere (ETIENNE & BERTHOULY, 2002), promoting photosynthetic competence and dehydration control (KOZAI & KUBOTA, 2001).

On the prior comparisons, no differences were noticed over the leaf area covered by stomata, but significantly lower values were observed on the stomata densities of the plants cultivated in liquid media (table 9), showing, once more, probably deleterious effects of

vermiculite on the plant's development, that presented larger but fewer stomata (figure 11 B and D). At the *in vitro* plants analyzed, the stomata distribution along abbatial and adaxial surfaces showed no differences in frequency in diaphonized leaves of the ones observed though scanning electron microscopy (SEM).

Table 9 - Means of stomata density (n./mm²), leaf area with stomata (mm² and %) and closed stomata at in vitro plants of A. xanthorrhiza cultivated under different conditions of availability of culture medium - semi-solid (Ss) and liquid (Lq) – and different gas exchange systems (hermetic gas exchange, passive through membranes and active in bioreactors);

	Stomata density (n./mm²)	Leaf area with stomata (mm²)	Leaf area with stomata (%)**	Closed stomata (%)**
Ss hermetic	306 a*	565,46 a	16,67% a	44,79% b
Lq hermetic	215 b	617,12 a	13,27% a	50,95% b
Ss gas exchange	349 a	450,30 a	15,72% a	75,26% a
Lq gas exchance	241 b	680,31 a	16,15% a	62,27% a
TI Twins Flasks	288 a	655,08 a	18,41% a	67,38% a
TI RITA	284 a	741,98 a	20,69% a	71,26% a
Average	*281*	*618,38*	*16,82%*	*61,99%*
CV%	*13,85%*	*18,07%*	*6,63%*	*11,45%*

* Means followed by the same letters in each of the analyzed variables do not differ according to the Scott-Knott mean separation test at 5% probability of error.

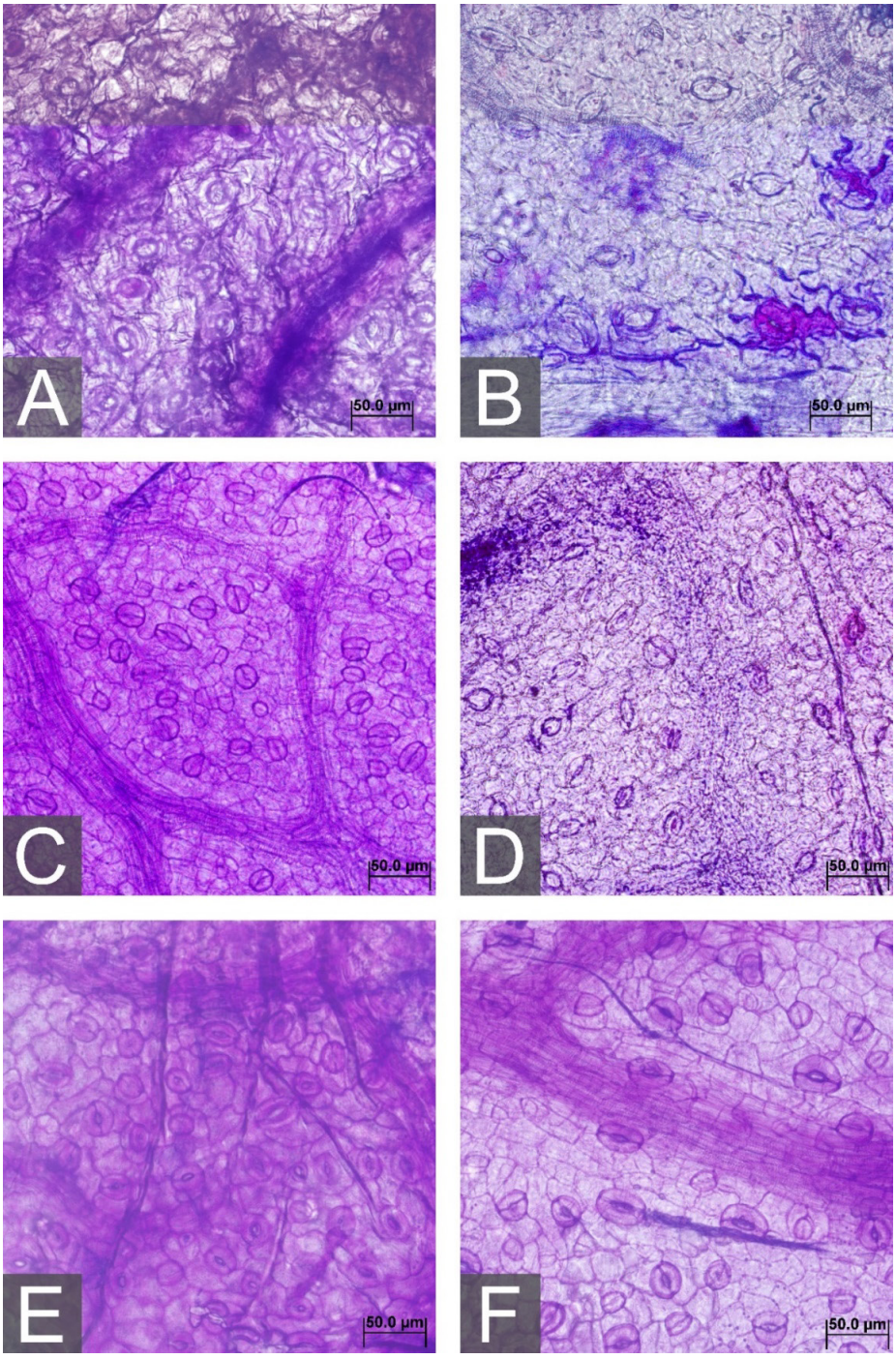
** Variable data transformed to arcosene [√ (yi + 0.05) / 100] during analysis

When analyzing the capability of response to environmental conditions, it was observed that the number of closed stomata was significantly higher in all treatments with gas exchange (figures 11 C, D, E, and F), showing and indicating the attribute of functional stomata (HAZARIKA, 2006) as expected.

The SEM images confirmed the pattern measured by diaphanization, also showing guard cells with a hypertrophic appearance (figure 12 A), some of which were raised equivalent to the epidermal tissue and presence of circular shapes, rather than elliptical shape, morphological indications of possible lower stomata functionality (DESJARDINS, HDIDER & DE RIEK. 1995; MOHAMED & ALSADON, 2010; QUIALA *et al*., 2012).

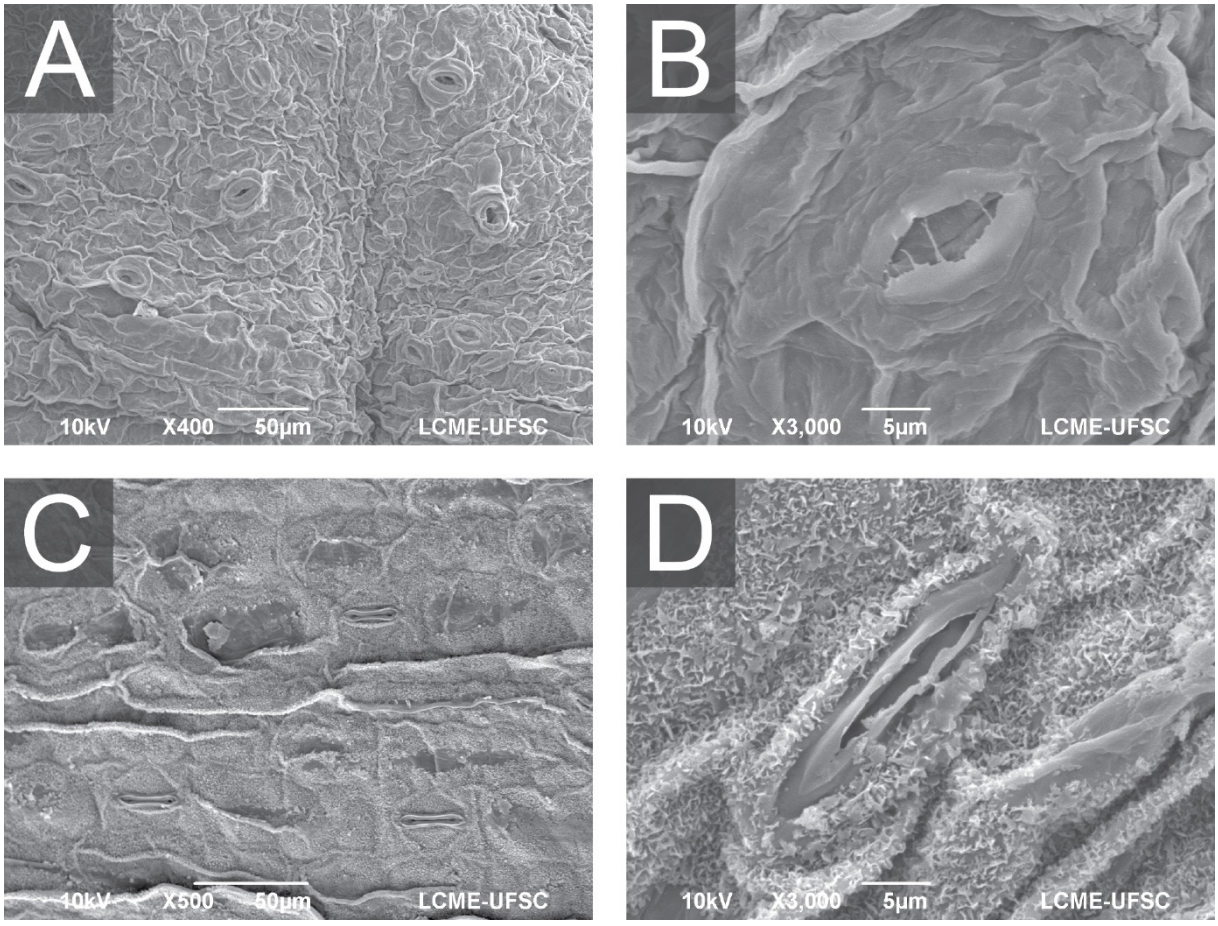
The nutrient-enriched medium – especially, with sucrose – and high relative humidity, tend to lead to poor stomatal regulation and malformations, along with thinner cell walls, lower cuticle and hair development, and wax deposition in the leaves, all factors that contribute to high vulnerability and mortality of micropropagated plants in *ex vitro* conditions (ZIV, 1991; GARVITA & WAWANGNINGRUM, 2020).

Figure 11 – Diaphanization of *A. xanthorrhiza* leaves for stomatal: A – gelled medium with no gas exchange; B – liquid medium and vermiculite with no gas exchange; C – semisolid medium with passive exchange; D – liquid medium and vermiculite with passive exchange; figure E – temporary immersion bioreactor with twin flasks; figure F – RITA temporary immersion bioreactor;



These factors tend to be turned over or alleviated through passive and forced GE (ETIENNE & BERTHOULY, 2002; KOZAI, 2010), as shown in the experiment data. In some cases, even plants with abnormal stomata formations and functionality patterns tend to recover in new formed organs (JIN *et al*., 2013). Those are worth data to be collected among different times o medium immersion, intervals, and aeration.

Figure 12 – Scanning electron microscope of *A. xanthorrhiza* leaves: A – general aspect and distribution of open stomata in plants cultivated in hermetic vessel; B – detail of an open stomata; C: general aspect and distribution of stomata in plants cultivated with temporary immersion bioreactor with twin flasks; D – detail of a closed stomata;



4.5 CONCLUSIONS AND FUTURE PROSPECTS

The passive GE and TI systems still did not show greater improvements for *A. xanthorrhiza* micropropagation, heading to propagation factors similar to those observed in

classic *in agar* cultures, and TI reinforced hyperhydration problems that were more subtle before, and incapable of generating rooted plants.

Nonetheless, GE and TI, demonstrated efficacy into inducing morpho-physiological adaptations of micropropagated plants to *ex vitro* condition, owing to promotion of normal and functional like stomata, not observed in hermetic culture conditions.

Further investigations on salts and sucrose reduction, balance between cytokinins and auxins in rooting phase to avoid senescence and, overall, aeration flux and immersion time reduction over plants morphogenesis and acclimatization may aggregated crucial bases for an industrial propagation protocol for the specie.

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5 CONSIDERAÇÕES FINAIS E PERSPECTIVAS

As melhorias propostas neste trabalho contornam ou superaram com sucesso alguns dos problemas típicos de cultura de tecidos de vegetais que também são encontrados na micropropagação de *A. xanthorrhiza*.

A suplementação com ampicilina na dosagem de 100 mg L^{-1} permitiu um método eficaz e de baixo custo para redução de contaminantes bacterianos e estabelecimento asséptico de 70% das culturas. O uso de AgNO3 na dosagem de 26 μM indica a promoção da emissão de novos brotos e folhas, conjuntamente a redução da senescência das plantas, potencializando os fatores de multiplicação e permitindo maior plasticidade no período de subcultura das plantas. O uso de mT a 1 μM satura a dose-resposta das plantas para brotos e emissão de brotos, e demonstrou três vezes mais atividade biológica e menores efeitos de inibição para emissão de raízes do que a comumente utilizada BAP.

Ainda existem muitas lacunas de conhecimento sobre a fidelidade genética e a capacidade de abarcar a diversidade genética existente nas espécies do protocolo de organogênese proposto. Nesse sentido, novos testes com raças tradicionais e linhagens são uma demanda para o futuro, assim como citometria de fluxo e análise genético-molecular das plantas regeneradas, buscando um alto rendimento garantido e um protocolo de regeneração *in vitro* fiel ao tipo.

Os sistemas passivos de trocas gasosas (GE) e biorreatores de imersão temporária (TI) ainda não apresentaram vantagens multiplicativas na micropropagação de *A. xanthorrhiza*, gerando fatores de propagação semelhantes aos observados em situações de cultivo clássicas (*in agar*), e TI reforçou problemas de hiperhidricidade e foram incapazes de promover a formação de raízes nas plantas. No entanto, GE e TI, demonstraram eficácia em induzir adaptações morfo-fisiológicas para condições *ex vitro*, devido à predominância de estômatos normais e funcionais, não observados em condições de cultivo hermético.

Investigações posteriores sobre a morfogênese e aclimatização das plantas cultivadas em TI com redução de sais e sacarose, novos balanços entre citocinina e auxina na fase de enraizamento para evitar senescência e, sobretudo, aumento no fluxo de aeração com redução do tempo de imersão podem agregar bases cruciais para um protocolo de micropropagação em larga escala desse importante recurso genético vegetal.

Como perspectiva geral, os avanços vislumbrados a partir dos dados encontrados lançam luz sobre as oportunidades do desenvolvimento de protocolos de cultura de tecidos para a espécie, uma vez que facilitam o estabelecimento, a manutenção e a multiplicação de propágulos de *A*

xanthorrhiza in vitro, tornando mais viável sua micropropagação, tanto para fins de propagação massal, quanto para atividades de pesquisa e investigação e variadas áreas de estudo da fisiologia e genética vegetal, para sua conservação em bancos de germoplasma *in vitro* e para o intercâmbio de germoplasma (figura 13).

Figure 13 – Infográfico com os principais problemas de pesquisa na micropropagação de *A xanthorrhiza*, soluções propostas, resultados positivos e negativos com as principais potencialidades e lacunas de conhecimento identificadas a partir da abordagem de investigação e análise.