WILSON VICENTE SOUZA PEREIRA

PERDA DA TOLERÂNCIA À DESSECAÇÃO EM SEMENTES DE *Copaifera langsdorffii* Desf. DE DIFERENTES AMBIENTES

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Engenharia Florestal, área de concentração em Ciências Florestais, para a obtenção do título de Doutor.

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LAVRAS – MG

2015

DEDICO...
A você que dedicou cada instante de sua vida aos seus filhos...
Por você que jamais mediu esforços pelo bem deles...
Porque nenhum sacrifício, nenhuma dificuldade, era grande demais para desistir deles.
A você devo cada uma de minhas vitórias.
A você devo até mesmo minha vida.
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É a você que dedico essa Tese.

À memória de minha avó Romana Alves Pereira.
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Walt Disney

RESUMO GERAL

Uma das características das sementes que representa um desafio para quem se propõe a trabalhar com conservação, é a sensibilidade à dessecação. Esta característica é encontrada em um grande número de espécies de interesse agronômico, florestal e medicinal. Sementes ortodoxas em processo germinativo têm sido utilizadas em estudos relacionados a este tema. Em geral, sementes ortodoxas apresentam perda da tolerância à dessecação após a protrusão radicular, entretanto, sementes de *Copaifera langsdorffii* perdem tal capacidade precocemente, se tornando sensíveis já na fase 2 da embebição. Assim, considerando a carência de estudos relacionados ao efeito do ambiente maternal sobre a tolerância à dessecação, o presente trabalho objetivou analisar aspectos moleculares e ecológicos da perda da tolerância à dessecação em sementes de *C. langsdorffii* em processo de germinação. As sementes foram coletadas em áreas de Cerrado *Stricto Sensu* em Montes Claros e Lavras, MG, e também em áreas de mata ciliar em Lavras, MG. As sementes das três procedências apresentaram perda precoce da tolerância à dessecação, contudo, sementes de Montes Claros, mantiveram a tolerância à dessecação por um período maior do que as outras duas procedências. Sementes do Cerrado de Montes Claros foram utilizadas para as análises bioquímicas e moleculares. Maiores alterações foram observadas em oligossacarídeos da família rafinósidica (rafinose, estaquiose e trealose), ao longo do processo de embebição, estando ausentes em estágios sensíveis à dessecação. Genes relacionados à germinação aumentaram sua expressão durante a germinação, enquanto aqueles ligados a tolerância à dessecação diminuíram, aumentando novamente após o tratamento de restabelecimento da tolerância à dessecação. Foi possível concluir, que há influência do ambiente materno na perda da tolerância à dessecação em sementes de *C. langsdorffii*, e que as concentrações, os oligossacarídeos da família rafinósidica, a síntese de proteínas resistentes ao calor e à expressão dos genes superóxido dismutase, ABA1, e proteínas de choque térmico, são correlacionadas com a perda e o restabelecimento da tolerância à dessecação de sementes desta espécie.

Palavras-chave: Copaíba. Expressão gênica. Efeitos ambientais. Genes de referência.

GENERAL ABSTRACT

One of the seed characteristics that represent a challenge for those who propose to work with conservation is the desiccation sensitivity. This characteristic is found in a large number of species of agronomic, forest and medicinal interest. Orthodox seeds in germination process have been used in studies regarding this theme. In general, orthodox seeds present loss of desiccation tolerance after radicle protrusion, however, *Copaifera langsdorffii* seeds lose such capacity precociously, becoming sensitive in phase 2 of imbibition. Therefore, considering the need for studies related to the effect of the maternal environment over the desiccation tolerance, the present work aimed at analyzing molecular and ecological aspects of the loss of desiccation tolerance in *C. langsdorffii* seeds in germination process. The seeds were collected from areas of *Stricto Sensu* Cerrado in Montes Claros and Lavras, MG, Brazil, as well as in areas of riparian forest in Lavras, MG. The seeds from all three origins presented precocious loss of desiccation tolerance, however, seeds from Montes Claros maintained desiccation tolerance for a longer period then the remaining origins. Seeds from the Cerrado of Montes Claros were used in the biochemical and molecular analyses. Greater changes were observed in oligosaccharides of the raffinose family (raffinose, stachyose and trehalose), over the soaking process, being absent in stages desiccation sensitive. Genes related to germination had their expression increased during germination while those related to tolerance to desiccation decreased, increasing again after the treatment of reestablishment desiccation tolerance. It was possible to conclude that there is influence of the maternal environment over the loss of tolerance to desiccation in *C. langsdorffii* seeds and that the concentrations of oligosaccharides of the raffinose family, the synthesis of proteins resistant to heat and the expression of the genes superoxide dismutase, ABA1 and heat resistant proteins is correlated with the loss and reestablishment of desiccation tolerance of seeds from this species.

Keywords: Copaiba. Gene expression. Environmental effects. Reference genes.

LISTA DE FIGURAS

SEGUNDA PARTE – ARTIGOS
ARTIGO 1

ARTIGO 2

ARTIGO 3

LISTA DE TABELAS

ARTIGO 1

ARTIGO 3

ARTIGO 4

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A tolerância à dessecação é normalmente definida como a capacidade de um organismo em suportar a secagem a níveis críticos e restabelecer o seu metabolismo normal após a reidratação. Esta habilidade está presente em espécies de todos os grupos de seres vivos, sendo que em vegetais, é mais comum em sementes, pólen e esporos. Quanto às sementes, existem três grupos classificados de acordo com a tolerância à dessecação: Ortodoxas (tolerantes), recalcitrantes (sensíveis) e intermediárias (com comportamento intermediário entre os dois primeiros grupos) (ELLIS; HONG; ROBERTS, 1990; ROBERTS, 1973).

Muitas espécies vegetais de interesse produzem sementes recalcitrantes, o que tem sido um grande desafio na tecnologia de sementes. Este tipo de semente não suporta secagem e, tampouco, o armazenamento às baixas temperaturas, tendo viabilidade e disponibilidade curta ao longo do ano. Tais características dificultam os estudos relacionados à sensibilidade à dessecação em sementes recalcitrantes. Por outro lado, sementes ortodoxas toleram a secagem e o armazenamento, apresentando maior facilidade para estudo. Além disso, durante o processo germinativo elas perdem a tolerância à dessecação, tendo sido proposto por Sun, Marzalina e Khoo (1999), o uso destas, em estudos relacionados à sensibilidade à dessecação.

Uma importante ferramenta no estudo dos mecanismos de tolerância à dessecação são os estudos moleculares. A avaliação das variações nas concentrações de açúcares, principalmente aqueles relacionados à tolerância à dessecação, bem como proteínas, são de grande auxílio na compreensão dos mecanismos de tolerância à dessecação. Além disso, com o estudo da expressão

gênica, pode-se compreender quais são as mudanças relacionadas com a perda e o reestabelecimento da tolerância à dessecação. Contudo, dadas aos possíveis erros e variações os quais podem ocorrer durante as etapas do estudo da expressão gênica, se faz necessário o uso de um fator de normalização. Para tal, a validação de pelo menos três de genes cuja expressão é uniforme dentre os tratamentos analisados é importante, sendo esses os genes de referência.

Copaifera langsdorffii é um importante representante do gênero *Copaifera* no Brasil, apresentando ampla distribuição geográfica. Estudos anteriores relacionados à perda da tolerância em sementes dessa espécie, durante a germinação, evidenciaram que a mesma perde a tolerância prematuramente quando comparada às demais espécies já estudadas (PEREIRA et al., 2014). Considerando o comportamento incomum da espécie escolhida, e sua presença em ambientes com características climáticas contrastantes, a mesma é interessante para auxiliar na elucidação dos fatores ligados à sensibilidade à dessecação.

Considerando tais fatores, no presente trabalho, objetivou-se analisar aspectos ecológicos e moleculares ligados a perda da tolerância à dessecação em sementes de *Copaifera langsdorffii* Desf, durante a germinação.

2 REFERENCIAL TEÓRICO

2.1 Tolerância à dessecação

Define-se tolerância à dessecação como a habilidade de um organismo em suportar a secagem a níveis críticos, sendo capaz de reestabelecer o seu metabolismo normal após a reidratação (ALPERT, 2000; OLIVER; TUBA; MISHLER, 2000). Esta habilidade está presente em todos os grupos de organismos, desde bactérias a animais. Em espécies vegetais, ocorre com maior frequência em sementes, pólen e esporos (ALPERT, 2005; HOEKSTRA, 2005). Para sementes, esta habilidade é de grande importância fitotécnica, pois permite a dispersão para longe da planta mãe, possibilitando assim a colonização de novas áreas.

Na biologia de sementes, existem três categorias básicas quanto à tolerância à dessecação e ao armazenamento. Sementes sensíveis à secagem são denominadas recalcitrantes. Estas não resistem ao armazenamento por períodos longos, bem como não toleram a secagem a níveis críticos. Sementes tolerantes, as quais são capazes de suportar a secagem a níveis inferiores a 10%, permanecendo ainda viáveis por longos períodos de armazenamentos são definidas ortodoxas (ROBERTS, 1973). O terceiro grupo, as sementes intermediárias, apresenta tolerância a secagem e armazenamento moderados (ELLIS; HONG; ROBERTS, 1990).

Sementes ortodoxas, ao longo do seu processo de desenvolvimento, atravessam duas etapas em que seu comportamento fisiológico é similar ao das recalcitrantes (PARCY et al., 1994). O primeiro é durante a formação na qual a semente está se desenvolvendo e ao final deste período (maturação) adquire a tolerância. O segundo é o processo germinativo, no qual a semente perde essa capacidade. Tanto as fases iniciais do processo de formação da semente, quanto

o processo de germinação, têm em comum a alta atividade metabólica. Este metabolismo está ligado ao desenvolvimento da semente para que ela seja dispersa (maturação) ou para germinar e formar a plântula (germinação). Durante a maturação, ocorre a ativação de mecanismos relacionados à tolerância à dessecação e redução dos demais processos, ocorrendo o oposto durante a germinação (KERMODE; FINCH-SAVAGE, 2002; NEDEVA; NIKOLOVA, 1997; PARCY et al., 1994).

Muitas espécies vegetais de interesse econômico, medicinal ou ecológico, produzem sementes de comportamento recalcitrante ou intermediário, bem como apresentam disponibilidade em períodos curtos ao longo do ano, sendo um dos maiores problemas na conservação de sementes e produção de mudas destas espécies. Dessa forma, é importante o conhecimento dos mecanismos ligados à tolerância/sensibilidade à dessecação, no entanto, tal estudo em sementes recalcitrantes é dificultado, dada as características destas. Por outro lado, considerando a perda da tolerância à dessecação em sementes ortodoxas em processo germinativo, Sun, Marzalina e Khoo (1999) propuseram o uso dessas sementes para a realização de estudos relacionados à sensibilidade à dessecação.

Considerando as três fases do processo de germinação de uma semente ortodoxa (BEWLEY et al., 2013), era postulado como regra que a perda da tolerância à dessecação ocorreria próximo à protrusão radicular (germinação). Isso foi evidenciado em várias espécies tais como *Medicago truncatula* (FARIA et al., 2005), *Sesbania virgata* (MASETTO et al., 2008), *Peltophorum dubium* (GUIMARÃES et al., 2011), *Solanum lycopersicum* e *Abelmoschus esculentus* (LIN; YEN; CHIEN, 1998). Contudo, foi observado em estudos anteriores, que *Copaifera langsdorffii* apresenta um comportamento diferente deste, iniciando a perda ainda na fase 1 da embebição, sendo a mesma perdida totalmente na metade da fase 2 (PEREIRA et al., 2014).

2.2 Mecanismos de tolerância à dessecação

Ao longo do processo de secagem, ocorre a concentração dos solutos no interior da célula, o que pode resultar na desnaturação de proteínas, perda da composição química, aumento do pH intracelular e danos na integridade de membranas e do DNA (NEDEVA; NIKOLOVA, 1997). Podem ainda ocorrer danos físicos causados não só pela saída de água, mas pela entrada durante o processo de reidratação (WALTERS et al., 2001; WOLFE; BRYANT, 1999). Além disso, durante a secagem, pode haver limitação na eliminação de metabólitos tóxicos, os quais podem causar maiores danos à estrutura celular (BUITINK; LEPRINCE, 2004; LEPRINCE et al., 1995, 2000; LEPRINCE; HOEKSTRA, 1998).

Dessa forma, os mecanismos de tolerância à dessecação devem manter a integridade celular, não só durante a secagem e no estado seco, mas também durante o processo de reidratação (PAMMENTER; BERJAK, 1999). Neste sentido, uma massiva alteração na expressão gênica da semente é observada durante a desidratação, sendo que para *Arabidopsis thaliana*, é alterada a expressão de cerca de 30% do genoma (ANGELOVICI et al., 2010). Sendo ainda que 21% desses genes têm a expressão alterada ao final do processo de maturação. Além disso, a redução do metabolismo é considerada chave para manutenção da integridade celular no estado seco. Com o metabolismo reduzido, a demanda por água e fontes de energia é menor, além da produção de espécies reativas de oxigênio.

Dentre todos os transcritos, aqueles ligados à síntese de ácido abscísico (ABA), são os mais importantes no estudo da tolerância à dessecação (BEWLEY et al., 2013). Grande parte dos mecanismos ligados à tolerância à dessecação são responsivos à presença do ABA, sendo ele, mencionado como

um dos principais hormônios ligados ao processo (BARBEDO; MARCOS FILHO, 1998; BARTELS, 2005; NAMBARA et al., 2000).

O acúmulo de reservas tais como as proteínas LEA (*Late embryogenesis abundant*) e oligossacarídeos da família rafinósidica (rafinose, estaquiose e trealose) e a sacarose, tem sido relacionado com tolerância à dessecação (BLACK et al., 1999). Proteínas LEA são sintetizadas normalmente ao final do processo de maturação e junto aos oligossacarídeos, tem função de manutenção da estrutura celular e da integridade das moléculas de DNA durante o estado seco. Os açúcares têm atuação ainda, como substitutos da água na célula, evitando assim, a lixiviação de compostos intracelulares e ainda mudanças na membrana plasmática durante o estado seco e durante a reidratação (LEPRINCE, 1993). Os carboidratos acumulados na célula tornam o citoplasma mais viscoso, condição referenciada como “estado vítreo” ou vitrificação (BUITINK; LEPRINCE, 2004), sendo comum em sementes ortodoxas. Neste estado, o metabolismo é reduzido, bem como a difusão de moléculas dentro da célula, condição na qual são reduzidas as reações que poderiam causar danos a integridade celular.

Além dos mecanismos de manutenção da estrutura celular, ainda é necessário o controle de espécies reativas de oxigênio, cuja eliminação se torna difícil no estado seco (KRANNER; BIRTIC, 2005; PAMMENTER; BERJAK, 1999). Moléculas oxidantes causam grandes danos à estrutura celular, sendo deletéria a falha de sistemas antioxidantes (FINKEL; HOLBROOK, 2000; KRANNER; BIRTIC, 2005; LALOI; APEL; DANON, 2004). Dentre os diversos metabolitos produzidos com função antioxidante, recebem destaque na biologia de sementes, a superóxido dismutase (SOD), catalase e outras peroxidases (NOCTOR; FOYER, 1998) as quais catalisam a conversão do íon superóxido em H_2O_2 para posterior conversão em O_2 e H_2O.

2.3 Influência ambiental sobre as características da semente

A influência das condições ambientais sobre o organismo vivo tem sido amplamente apresenta em todas as classes de organismos. Para espécies vegetais, as condições tais como disponibilidade hídrica, fertilidade do solo, comprimento do dia e temperatura tem influência sobre o fenótipo da planta e também das sementes e pólen (DELPH; JOHANNSSON; STEPHENSON, 1997; FENNER, 1991; WEINER et al., 1997). Dependendo do ambiente parental, tanto a morfologia quanto a fisiologia de uma semente, são alteradas, sendo relatadas até mesmo diferenças quanto ao grau de dormência na mesma.

Quanto à tolerância à dessecação, vários estudos têm sido apresentados evidenciando mudanças no comportamento fisiológico das sementes em função do ambiente de origem. Daws et al. (2004a), mostraram que para *Aesculus hippocastanun*, as condições de temperatura do ambiente maternal influenciaram os níveis de sensibilidade à dessecação nas sementes. Daws et al. (2006), mostraram ainda que sementes de *Acer pseudopalatanus* produzidas em regiões de temperatura mais alta apresentam menor sensibilidade à dessecação, do que aquelas produzidas em locais mais frios. Sementes de *Camelia sinensis* oriundas do Japão suportaram a secagem e armazenamento por 6 anos, perdendo apenas 13% da viabilidade (AMMA; WATANABE, 1985), enquanto que aquelas provenientes da África do Sul foram classificadas como recalcitrantes por Berjak, Vertucci e Pammenter (1993).

Quanto a perda da tolerância à dessecação em sementes ortodoxas, não existem estudos que evidenciam alterações em tal comportamento, em função do ambiente maternal. Considerando as mudanças já observadas em espécies sensíveis à dessecação (AMMA; WATANABE, 1985; BERJAK; VERTUCCI; PAMMENTER, 1993; DAWS et al., 2004b, 2006), o estudo do efeito das

condições do ambiente maternal sobre a perda da tolerância à dessecação em uma semente ortodoxa, pode auxiliar na compreensão do processo.

2.4 *Copaifera langsdorffii*

Dentre os representantes do gênero *Copaifera*, *C. langsdorffii* é o que recebe maior destaque no Brasil (GUERRA; MEDEIROS FILHO; GALLÃO, 2006; VEIGA JUNIOR; PINTO, 2002). Essa espécie apresenta ampla distribuição no Brasil, sendo encontrada em áreas de caatinga, cerrado e mata atlântica no Nordeste (Bahia), Centro-Oeste (Goiás e Mato Grosso do Sul) e Sudeste (Minas Gerais e São Paulo) (QUEIROZ; MARTINS-DA-SILVA; COSTA, 2014).

Assim como nos demais representantes do gênero, é extraído do tronco de *C. langsdorffii*, óleo de interesse medicinal (VEIGA JUNIOR; PINTO, 2002), com aplicações antiinflamatórias, bactericida, diurético e expectorante (FREIRE; BRITO-FILHA; CARVALHO-ZILSE, 2006). Além disso, a madeira é utilizada na construção civil, peças torneadas, cabos de ferramentas e vassouras, até mesmo portas e painéis (LORENZI, 2002; VEIGA JUNIOR; PINTO, 2002), sendo a espécie ainda utilizada na arborização rural e urbana (JELLER; PEREZ, 1997) e na recuperação de áreas degradadas.

Sementes de *C. langsdorffii* apresentam comportamento ortodoxo (HONG; ELLIS; LININGTON, 1998), sendo referenciadas como apresentando dormência ocasional (FOWLER; BIANCHETTI, 2000; LIMA et al., 2008), estando esta ligada ao impedimento na absorção de água, bem como a presença de inibidores da germinação. Quanto à tolerância à dessecação, foi evidenciado comportamento fisiológico incomum em relação às demais sementes ortodoxas estudadas (PEREIRA et al., 2014). Sementes de *C. langsdorffii* se tornam sensíveis à dessecação já na fase 2 do processo de embebição, sendo a primeira

espécie ortodoxa descrita a apresentar tal comportamento. Dado a este comportamento e a importância dos estudos de tolerância à dessecação, bem como a presença da espécie em áreas de disponibilidade hídrica contrastantes, o estudo da perda da tolerância à dessecação em *C. langsdorffii* em função do ambiente, poderá gerar informações interessantes para a elucidação do problema.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 Environmental effects on *Copaifera langsdorffii* seeds: morphological and physiological aspects

VERSÃO PRELIMINAR submetida à revista *Brazilian Journal of Botany*, podendo sofrer alterações de acordo com o corpo editorial da revista.

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ABSTRACT: Maternal environment influences all morphological and physiological characteristics of an organism. Thus, this study aimed to characterize morphological and physiological differences of *Copaifera langsdorffii* seeds from three provenances. Fruits and seeds were collected in Cerrado areas in Montes Claros, MG and Lavras, MG, and riparian forests in Lavras, MG. Size, mass and water content of fruits, seeds and aril were measured, being also evaluated physiological characteristics (imbibition, germination and seedling size). There are differences at the seed size, imbibition, speed and percentage germination among the seed provenances. The data shows that the maternal environment influences both morphological and physiological characteristics of *Copaifera langsdorffii* seeds.

Key-Words: Copaiba, environmental effect, seeds.

Introduction

From the large number of species on the *Copaifera* genus, *Copaifera langsdorffii* is the most representative. The species have wide geographic distribution in Brazil, being present in Brazilian northeast (Bahia), Center west (Goiás and Mato Grosso do Sul) and southeast (Minas Gerais and São Paulo) (Andrade Júnior & Ferraz, 2000). As the other species from the genus, from the wood of *C. langsdorffii* can be extracted oils with medicinal applications (Freire *et al.*, 2006; Veiga Junior *et al.*, 2007)**.**The wood can be used for buildings, furniture, tools and many another purposes (Lorenzi, 2002; Veiga Junior & Pinto, 2002). Also, the trees are used for degraded areas recovery and also urban and farm afforestation (Jeller & Perez, 1997; Lorenzi, 2002).

The biometrics knowledge is an important factor for understanding the germination process, vigor, storage and propagation methods. Biometrics also is important for the differentiation of species and characterization of ecological aspects of the plant (Matheus & Lopes, 2007). Factors as the day length, temperature, water availability and herbivore attack on where the plant grows have large influence on its phenotypic characteristics (Fenner, 1991; Delph *et al.*, 1997; Weiner *et al.*, 1997). Thus, the seed characteristics are also influenced by the environment, and affect the plant vigor, also its establishment and growth (Daws *et al.*, 2004).

The biometrics studies can helps on the understanding of the plant ecology and can shows the effects of the maternal environment on the species. Furthermore, this knowledge can help in the seedling production, aiming its use for reforestation projects. Then, this study aimed to evaluate biometrics and physiological differences on *C. langsdorffii* seeds in relation to the collection area.

Materials and methods

Plant Material

Plant material was collected from *C. langsdorffii* trees on *stricto sensu* Cerrado in the counties of Montes Claros and Lavras (Minas Gerais, Brazil) and also in a riparian forest in county of Lavras between July and August 2011. To morphological assays fruits were collected before its openingand to physiological assays seeds were collected after dispersion (open fruits). To physiological assays seeds were cleaned by manual removing of the aryl and dried into storing water content (10 ± 2% at wet basis) on climate-controlled chamber [20 °C, 60% air relative humidity (RH)]. After drying, the seeds were stored in sealed semipermeable plastic bags in a cold chamber (5 ºC, 40% RH), until the beginning of the experiment. For each environment, the experiments were carried separately and the experiments were initiated within 1 week from the collection date.

Characterization of fruits and seeds

Fruits were measured using a digital paquimeter about the length (bigger measurement), width (smaller measurement) and thickness. However, seeds have different shapes, thus, only width (parallel to the hilum) and thickness were measured. Moreover, the water content was determined for fruits, aryls and seeds (separately) by oven drying at 103 °C for 17 hours as adapted from Ista (2004). For both fruits and seeds biometrics were used 50 replicates of individual fruits or seeds.

As standard, the germination tests for *C. langsdorffii* seeds were preceded with mechanical scarification with sandpaper and conditioning at paper roll at 25°C with constant light. It was evaluated the germination and normal seedlings formation, being considered normal the seedling with all health structures (leaves, stem and primary root). Four replicates of 25 seeds each were

used for germination test which was evaluated at. Also the germination speed index (GSI) was calculated by the formula proposed by (Maguire, 1962). After 40 days, the width of the stem and primary root and the diameter on the interface root/stemwere measured individually. For these measures were utilized 30 seedlings sampled randomly of each provenance.

Effect of the C. langsdorffii seed leached under lettuce seed germination

The effect of the *C. langsdorffii* seed leachate of each provenance on lettuce seed germination (*Lactuca sativa*). For that, 70 grams of seeds of each provenace were immersed on the same weight of distilled water during 24 hours. The resulting solution was used to moisten the germination paper used as substrate for lettuce seed germination. The seeds were conditioned into Petri dishes at 20°C and constant light being used distilled water as a control. For the germination test were used four replicates of 100 seeds each.

Effect of pyroligneous extract and provenance under C. langsdorffii seed germination

The effect of pyroligneous extract under the germination of the three seeds provenances was also accessed. For that, we prepared the extract by gas recovery from eucalyptus wood burning at 550°C. The extract was distilled two times in a rotatory evaporator at 100 °C. The extract for tests were diluted at 1/50 and 1/5000. The seeds (without scarification) were immersed in the extract for 48 hours and immediately after treatment, was performed the germination test, as described above. The criteria to assessing germination on this assay were formation of normal seedlings, being used 4 replicates of 25 seeds on each sample.

Data analysis

The experimental design was completely randomized for all assays. The data analysis was carried by ANOVA and if there is statistical influences of the provenance on the evaluated characteristics, was done the Tukey's test (5% probability) by the software R for Windows (RCoreTeam, 2014). Also, for complementary data the environmental conditions between the provenances, the climate data from the region of Montes Claros (Federal University of Minas Gerais climate station) and Lavras (Federal University of Lavras climate station) were collected.

Results

Characterization of fruits and seeds

The three provenances have not difference about the fruit size. However, fruits of the Cerrado from Lavras had higher fresh and dry mass, and for these characteristics, fruits of the Cerrado from Montes Claros had the lowest values (Table 1). There was no difference between the provenances about the fruit water content. In relation to the seed aryl, the higher fresh and dry mass was observed on seeds from Lavras Cerrado, and lower from seeds of Montes Claros Cerrado (Table 2).

Table 1. Morphological variations of fruits of *Copaifera langsdorffii* from different environments

	Fresh mass (g/fruit)	Dry mass (g/fruit)	Water content (%)	Lenght (mm)	Thickness (mm)	Width (mm)
Cerrado (Montes Claros)	0.489c	0.443c	9.30a	21.01a	11.85a	21.01a
Cerrado (Lavras)	1.124a	0.975a	13.14a	25.53a	14.91a	22.53a
Riparian (Lavras)	0.926b	0.841b	9.45a	22.12a	14.22a	22.12a
p	<0.0001	<0.0001	0.83	0.39	0.42	0.068

Same letters on the columns indicates absence of differences among the provenances by the Tukey test at 5% of probability.

Table 2. Variations on the aryl of *Copaifera langsdorffii* seeds from different provenances

	Fresh Mass (g/seed)	Dry Mass (g/seed)	Water (%)
Cerrado (Montes Claros)	0.223c	0.185c	17.32c
Cerrado (Lavras)	0.432a	0.333a	22.29a
Riparian(Lavras)	0.322b	0.260b	19.17b
p	<0.0001	<0.0001	0,02

Same letters on the columns indicates absence of differences between the provenances by Tukey test at 5% of probability.

In relation to the seed measurements, the provenance influenced the thickness, fresh and dry mass (Table 3). Higher values were observed in seeds of Cerrado from Lavras. The water content on seeds of Cerrado from Montes Claros had the lowest value.

Table 3. Morphological variations in *Copaifera langsdorffii* seeds from different environments.

	Width (mm)	Thickness (mm)	Fresh Mass (g)	Dry Mass (g)	Water (%)
Cerrado (Montes Claros)	8.40a	6.42c	0.325c	0.293c	10.12c
Cerrado (Lavras)	12.00a	8.80a	0.592a	0.505a	14.53b
Riparian (Lavras)	10.46a	8.06b	0.450b	0.382b	15.77a
p	0.08	<0.0001	0.0002	0.0025	<0.0001

Same letters on the columns indicates absence of differences between the provenances by Tukey test at 5% of probability.

The seeds of the three provenances shown similar germination (p = 0.18; CV = 8.15) and formation of normal seedlings (p = 0.206; CV = 9.55) (Figure 1A). However, the germination speed index of seeds from Montes Claros Cerrado had higher value than the seeds of other provenances (p< 0.0001; CV = 13.81) (Figure 1B).

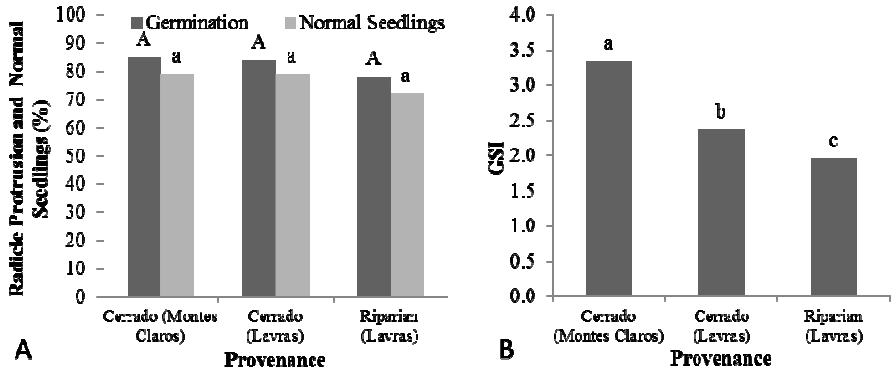

Figure 1. Germination and normal seedlings percentage (A) and germination speed index (GSI) (B) of *Copaifera langsdorffii* seeds in relation to the seed provenance. Same letters indicate no significant differences between the seed provenances, according to a Tukey test ($p \leq 0.05$).

The visible germination (radicle protrusion) starts, for all provenances, at the sixth day. At this time 53% of Montes Claros Cerrado seeds had germinated while the other provenances reached this value after 8 (Lavras Cerrado) or 9 (Riparian) days. In relation to the seedlings size, seeds from Montes Claros had shown the highest values. There wereno differences among the provenances regarding the root length and stem diameter (Table 4).

Table 4. Biometric variations in seedlings of *Copaifera langsdorffii* developed from seeds from different provenances.

	Diameter* (mm)	Total Length (cm)	Root length (cm)	Stem Length (cm)
Cerrado (Montes Claros)	2.62a	13.96a	8.04a	5.91a
Cerrado (Lavras)	2.79a	13.15ab	7.80a	5.35ab
Riparian (Lavras)	2.75a	11.85b	6.90a	4.92b
p	0.24	0.012	0.08	0.012

Same letters on the columns indicates absence of differences between the provenances by Tukey test at 5% of probability. *Diameter evaluated on the interface root/stem.

Effect of the C. langsdorffii seed leached under lettuce seed germination

There was no effect of the seed leached over the germination of lettuce seeds (p = 0.76; CV = 0.11) (Figure 2A). However, the seed leached affected the GSI, being the leached of seeds of Cerrado from Montes Claros resulted in the lowest GSI when compared to another seed provenances and the control (p = 0.00001; CV = 20.13)(Figure 2B).

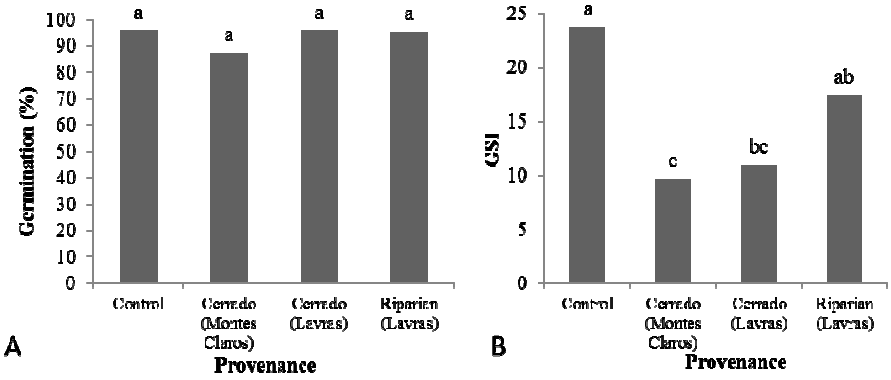

Figure 2. Germination percentage (A) and germination speed index (GSI) (B) of lettuce (*Lactuca sativa*) seeds under effect of the seed leached of three provenances of *Copaifera langsdorffii*. Same letters indicate no significant differences between the seed provenances, according to a Tukey test ($p \leq 0.05$).

Pyroligneous extract on seed germination

The normal seedlings developed from *C. langsdorffii* seeds showed differences under effect of the pyrolegneous extract. For the three provenances, the high concentration of the extract resulted in the lowest normal seedlings percentage. On the other hand, seeds from Lavras (Cerrado and Riparian Forest) had highest germination when submitted to the low concentration of the extract, differently of the seeds from Montes Claros Cerrado, that had higher germination when were not submitted to the extract (Figure 3).

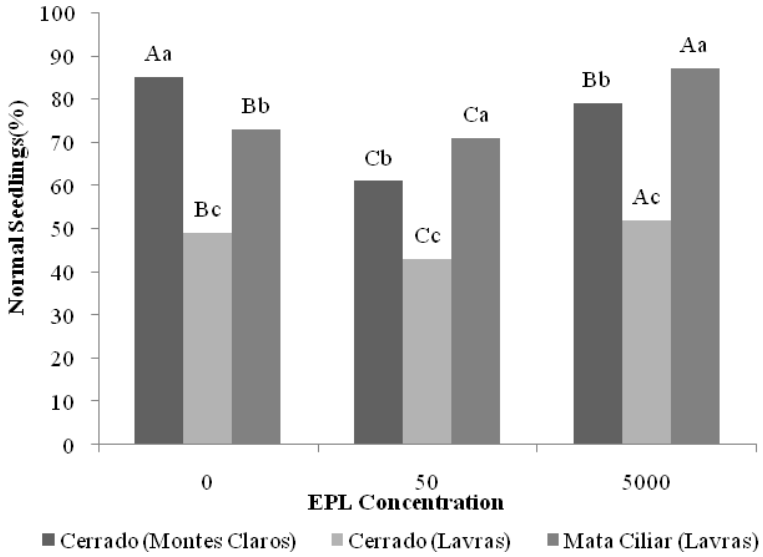

Figure 3. Response of three *C. langsdorffii* seeds provenance to the EPL concentrations. Same uppercase letters indicate absence of differences between EPL in each provenance. Same lowercase letters indicates absence of differences between the seed provenances in each pyrolegneous extract concentration.

The climate data can be observed on the figure 4. The fruit development occurs between March to June being the seed dispersion is during July to September (Freitas & Oliveira, 2002; Lorenzi, 2002; Pedroni *et al.*, 2002).

Discussion

Gutterman (2000) reported that the environment and even the position of the seeds on the tree can influence its characteristics. The three provenances had major differences about the water availability, being the Cerrado from Montes Claros driest provenance. The environmental effect under the characteristics between the provenances was reported already not only physically, but also physiologically (Wulff, 1986; Fenner, 1991; Tompset & Pritchard, 1993; Delph *et al.*, 1997; Weiner *et al.*, 1997).

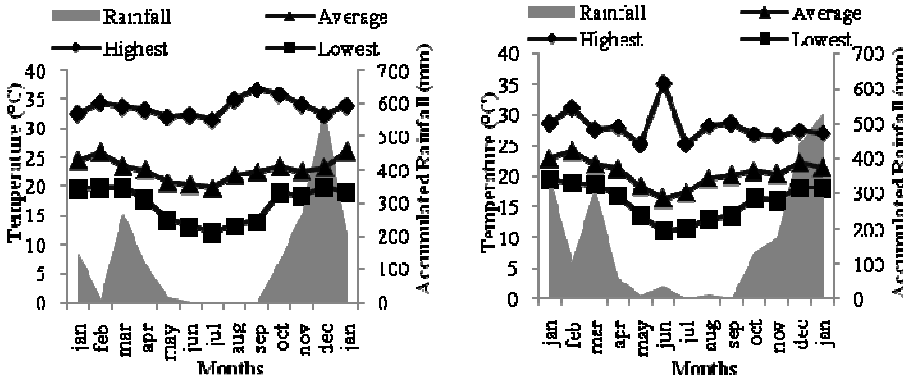

Figure 4. Cilmate data from the region of Montes Claros (A) and Lavras (B). Data obtained from (A) Climate Station of the Agronomic Institute, Federal University of Minas Gerais, campus of Montes Claros. (B) Climate Station of Federal University of Lavras.

High temperatures results on reduction of seed size and mass (Fenner, 1991; Agrawal *et al.*, 2005), and the region of Montes Claros had, during the seed development, higher temperatures and lower rainfall than Lavras. Gutterman (2000) mentions that, despite the effect of the environment under the germination is real, there is no a standard, thus, this response depends of the species. Seed morphological variations of the different provenances were reported for *Senna spectabilis* (Souza *et al.*, 2007), that had the seed size and production influenced by the environmental conditions.

The reduction of the lettuce GSI under the *C. langsdorffii* seed leached shows the presence of germination inhibitors already reported for the species (Buckeridge *et al.*, 2000). Once the GSI decrease more on the effect of extract from the Montes Claros Cerrado seeds than the others, this may be due to the higher inhibitor concentration on this provenance.

The effect of the pyrolegneous extract on seed germination is reported by many researchers (Brown *et al.*, 1994; Pierce *et al.*, 1995). Brown & Staden (1997) had reported the inhibitory effect of the high concentrated extract on seed

germination, which can be toxic for the seeds, as observed on this study and another. Also, the increasing on the germination under the treatment with the low concentrated extract was also observed (Brown *et al.*, 1994; Brown & Staden, 1997; Dayamba *et al.*, 2008). However, on the present study, was also observed that, for a species the response to the extract can be influenced by the provenance of the seeds. The stimulant effect of the low concentration extract could be observed on both provenances from Lavras, but seeds from Cerrado of Montes Claros had better germination under the control. The first one had more germination without the extract and the other two provenances had an increase on the germination under the effect of low extract concentrations. This shows that also the response to the extract can be differential by influence of the environment.

Conclusions

Environmental conditions influenced the morphological and physiological characteristics of *Copaifera langsdorffii* seeds.

The response of *C. langsdorffii* seeds to the pyrolegneous extract can be influenced by the environment.

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(VERSÃO PRELIMINAR)

ARTIGO 2 Is the loss of desiccation tolerance affected by maternal environment?

VERSÃO PRELIMINAR submetida à revista *Seed Science Research*, podendo sofrer alterações de acordo com o corpo editorial da revista.

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Running head title: Desiccation tolerance on *Copaifera langsdorffii*

Abstract: There is little information about the influence of the environment on the seed morphological and physiological characteristics, especially on desiccation tolerance. Therefore, the objective of this paper was to study the desiccation tolerance of *Copaifera langsdorffii* seeds from different provenances. Seeds were harvested in Cerrado *stricto sensu* in Montes Claros, Cerrado *stricto sensu* and riparian forest in Lavras. These cities are located, respectively, in the north and south of the State of Minas Gerais, Brazil. The climate in the north of the State shows higher temperatures and lower precipitation than the south. For each provenance, the imbibition curve was described by measuring the increase in the seed water content along the incubation. The seeds were then imbibed at different times and submitted to drying with posterior rehydration in order to assess survival (desiccation tolerance). Additionally, for seeds from Montes Claros, the effect of pre-humidification and scarification on desiccation tolerance was assessed. It was

observed that seeds from the Cerrado of Montes Claros are more desiccation-tolerant than those from Lavras, pointing out that the maternal environment influences desiccation tolerance. Pre-humidification and scarification did not influence desiccation tolerance.

Keywords: *Copaifera langsdorffii*, copaiba, desiccation tolerance, maternal effect.

Introduction

The most usual definition for desiccation tolerance is the ability to survive to almost complete water losses, being able to recover the normal metabolism after rehydration (Oliver *et al.*, 2000; Hoekstra *et al.*, 2001). Alpert (2000) also defines desiccation tolerance as the ability to equilibrate the internal water content with the moderately dry air and restore the normal metabolism after rehydration. This ability is present in all groups of organisms, from microorganisms to animals (Alpert, 2000). In plants, mostly in seeds, pollen and spores (Hoekstra, 2005), structures that must be able to disperse far from parental plants, and it is necessary to be dry for a certain period. There are three groups of seeds, based on desiccation tolerance: those tolerant to drying, cold temperatures and storage for long periods (orthodox), those which do not survive to drying, cold temperatures and storage (recalcitrant) (Roberts, 1973), and those which can survive to moderate drying and cooling and can be stored for periods longer than those belonging to the recalcitrant group, but smaller than the orthodox (intermediate) (Ellis *et al.*, 1990).

During their development, orthodox seeds alternate phases of tolerance and sensitivity to desiccation. During embryo formation and growing, while metabolism and water are high, seeds are sensitive to desiccation (Parcy *et al.*, 1994). During the maturation phase, seeds become tolerant, keeping this way until dispersion. When seeds are imbibed and the germination process starts,

desiccation tolerance is progressively lost (Nedeva & Nikolova, 1997). Considering this, and the difficulties in studying desiccation sensitivity in recalcitrant seeds, Sun *et al.* (1999) propose the use of germinating orthodox seeds to understand desiccation sensitivity.

During the germinative process, the metabolism is reactivated, reserve consumption starts and desiccation tolerance mechanisms are deactivated (Kermode & Finch-Savage, 2002). Based on the three phases of germination (Bewley *et al.*, 2013), most orthodox seeds keep desiccation tolerance during phase 1 and start to lose it by the end of phase 2. Among the orthodox seeds which have already been reported in the literature, desiccation tolerance is lost when the radicle protrudes, and this behavior is associated to the intense metabolism in phase 2. On the other hand, a different behavior concerning desiccation tolerance has been reported for *Copaifera langsdorffii* (Pereira *et al.*, 2014). Its seeds start losing their desiccation tolerance in phase 1 and, by the middle of phase 2, it is completely lost.

It has been already reported that the environment has a great influence on seed characteristics. Phenotypic and physiological characteristics of the seeds are influenced by maternal environment conditions, such as day length, temperatures, water availability, soil characteristics and even the action of herbivores (Wulff, 1986; Fenner, 1991; Delph *et al.*, 1997). These characteristics also influence seed vigor, germination, as well as seedling establishment and growing (Stearns, 1960; Daws *et al.*, 2006). Some authors reported the effect of maternal environment temperatures under seed dormancy (Fenner, 1991; Pritchard *et al.*, 1999). Desiccation sensitivity is also influenced by climatic factors, as observed for *Aesculus hippocastanum* and *Acer pseudoplatanus*; therefore, it is important to consider the environmental influence on the response to desiccation by the seeds (Daws *et al.*, 2004; Daws *et al.*, 2006). However, these factors have not been well explored, and reports on the effect of the

environment on desiccation tolerance are scarce in the literature (Tompset & Pritchard, 1993; Finch-Savage & Blake, 1994).

Since *C. langsdorffii* can be found in different environments (Queiroz *et al.*, 2014), understanding the loss of desiccation tolerance in *C. langsdorffii* can be useful to explain the uncommon behavior observed for this species. Furthermore, this study can be useful for the comprehension of the effects of the maternal environment under seed characteristics. Therefore, the objective of this study was to characterize the loss of desiccation tolerance in *C. langsdorffii* seeds from different provenances and pre-germinative procedures.

Methods

Copaifera langsdorffii seeds were collected after dispersion (open fruits) at Cerrado *stricto sensu* in Montes Claros and in Lavras, MG, as well as in riparian forests in Lavras, MG. After collection, the seeds were dried to 10±2% water content and stored in semipermeable bags in a dry and cold chamber (40% air relative humidity / 5°C), until the experiments were set.

Assay 1 – Loss of desiccation tolerance

The assay on the loss of desiccation tolerance was carried out separately for each provenance. Therefore, a sample of 30 seeds was mechanically scarified using sandpaper, imbibed in paper at 25°C/constant light, and had the weight measured at regular times. Using this data, the imbibition curve was established for the determination of sampling points for desiccation tolerance tests. For each sample, points at phases 1 and 2 of the imbibition curve were chosen.

Another sample of mechanically scarified seeds was then imbibed as described above until they reached the chosen points. For each point, seed weight and water content were measured; the seeds were then placed in gerbox with silica gel until they reached the initial water content (variable as a function

of each provenance). Therefore, seed weight was measured at regular times until it reached the target weight, calculated by the use of Equation 1 (Hong & Ellis, 1996). After reaching the initial water content, they were maintained in such state for 72 hours. The seeds were subsequently submitted to pre-humidification at 100% (air relative humidity) and 25°C under constant light during 24 hours, and then imbibed in paper at 25°C under constant light.

$$Target\ weight = \left(\frac{(100 - Initial\ moisture\ content)}{(100 - Target\ moisture\ contet)}\right) - Initial\ weight$$

Equation 1 – Formula used for the calculation of the target seed weight during drying.

Assay 2 – Effects of pre-germinative treatments on desiccation tolerance

For this assay, only seeds from Montes Claros were used. The influence of mechanical scarification on desiccation tolerance was tested. Based on the data of assay 1, seeds were imbibed until reaching two distinct water content values, the first one being that in which seeds are still desiccation-tolerant, and the second one when seeds have already lost desiccation tolerance. Since non-scarified seeds present an uneven imbibition, each seed was weighed separately during imbibition until reaching the target water content (based on Equation 1). The seeds were then submitted to the drying process, as used in assay 1 for scarified ones. A germinative test was also carried out with seeds scarified or not, for comparison; however, these samples were not submitted to the imbition/drying cycle. The germination criteria were the formation of normal seedlings. The loss of desiccation tolerance in relation to water content was analyzed, comparing scarified seeds to those not scarified.

The loss of desiccation tolerance in seeds submitted to pre-humidification was also analyzed. In this case, an imbibition curve was made using mechanically scarified (sandpaper) seeds submitted to pre-humidification

at 100% air, relative humidity at 25°C and constant light. Subsequently, the seeds were conditioned in paper at the same temperature and light conditions for the same times used for the samples from Montes Claros mentioned in assay 1. The seeds were then dried in silica gel until reaching the initial water content and maintained in this state for 72 hours, when they were submitted again to pre-humidification and conditioned in paper at 25°C and constant light.

Data analysis

For all assays, desiccation tolerance was evaluated by germination, and the formation of normal seedlings were used as criteria. In each germination test, 4 replicates with 25 seeds were used. To describe desiccation tolerance, each environment or treatment was analyzed separately. Data were analyzed by Generalized Linear Models (GLM) by the Binomial family and the explanatory equation was established. The analysis was carried out using the software R for Windows 3.1.0 (RCoreTeam, 2014).

Results and discussion

For all provenances and treatments, despite differences in the times, the same behavior was observed regarding the loss of desiccation tolerance. Seeds become completely sensitive to desiccation by the middle of imbibition phase 2, as described for the species in previous research (Pereira et al, 2014). However, changes between the provenances were observed. Seeds from the Cerrado of Montes Claros kept desiccation tolerance until the end of phase 1, with a fast decrease upon the start of phase 2 (Figure 1A), when desiccation tolerance reached less than 5%. Seeds from the Cerrado and riparian forest of Lavras presented a similar behavior (Figures 1B and C), since desiccation tolerance started to decline at the beginning of phase 1, and was lost around the middle of phase 2.

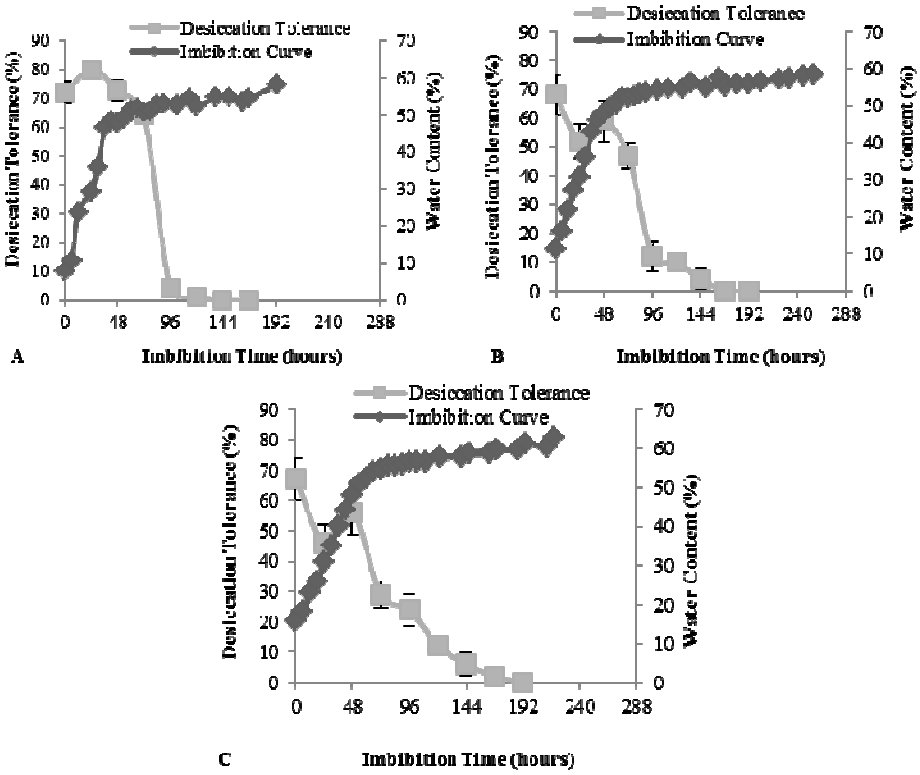

Figure 1. Loss of desiccation tolerance in scarified *C. langsdorffii* seeds, measured by the formation of normal sedlings, of seeds from Montes Claros Cerrado**(A)**, Lavras Cerrado **(B)** and Lavras riparian forest **(C)**.

Figure 2 shows the loss of desiccation tolerance in seeds from the Cerrado of Montes Claros which were submitted to pre-humidification before the beginning of imbibition. When the loss of desiccation tolerance is compared with those seeds that were not submitted to pre-humidification (Figure 1A), it is possible to observe that there is a small change in behavior. With pre-humidification, the loss starts after the beginning of phase 1 (that happens in phase 2 without pre-humidification), and the decrease is smaller and slower for seeds submitted to pre-humidification. However, in both cases, there is not more

desiccation tolerance after the middle of phase 2, as observed in all cases studied in this paper.

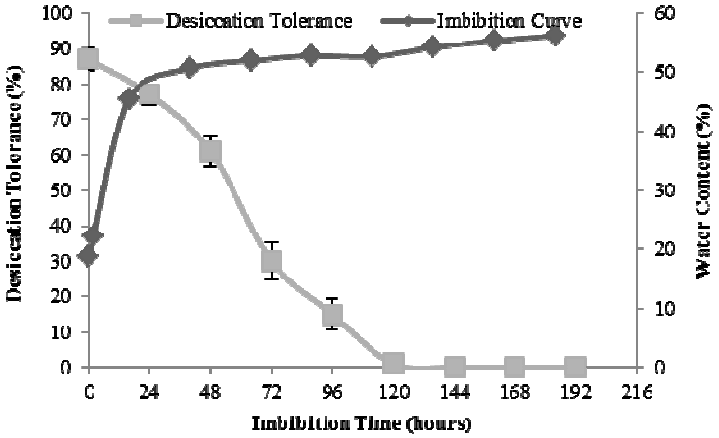

Figure 2. Loss of desiccation tolerance in scarified *Copaifera langsdorffii* seeds submitted to the pre-humidification treatment. The x axis represents hours of imbibition after the treatment, and time 0 represents seeds submitted to the treatment, but not to the drying process (control).

Scarified seeds take 24 or 96 hours to reach the water content of 24 (desiccation-tolerant) or 55% (desiccation-sensitive), respectively. However, non-scarified seeds have not shown uniformity in imbibition, taking from 48 to 72 hours to reach a water content of 24%, or 96 to 120 hours to reach 55%. The germination speed was also different between the treatments. Scarified seeds reached 82% of germination at 192 hours of imbibition; at the same time, only 25% of non-scarified seeds had germinated. It takes around 384 hours for non-scarified seeds to reach the final radicle protrusion percentage that is reached in scarified seeds (Figure 3).

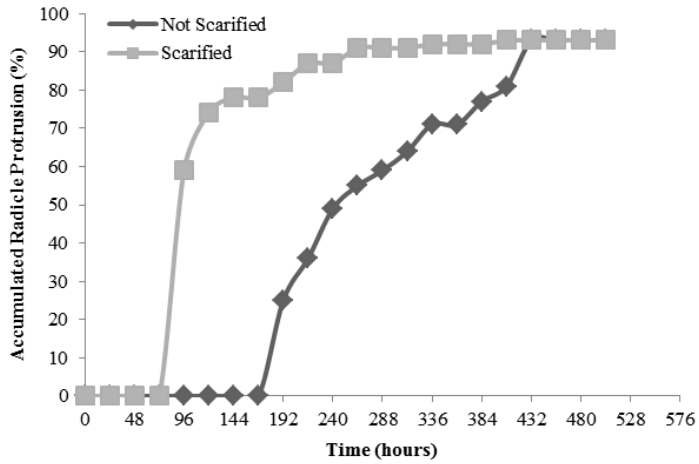

Figure 3. Accumulated germination of *Copaifera langsdorffii* seeds under the influence of the pre-germinative treatment.

The loss of desiccation tolerance is not influenced by scarification, as shown in Figure 4 ($p>0.05$). Scarified seeds are still desiccation-tolerant at a water content of 24%, and not at 55%. However, non-scarified seeds start losing their desiccation tolerance with a water content of 24%. At this water content, desiccation tolerance is higher in scarified seeds than in those non-scarified, which shows a decrease in the beginning of imbibition. At a water content of 55%, most seeds are desiccation-sensitive, independently of the scarification treatment.

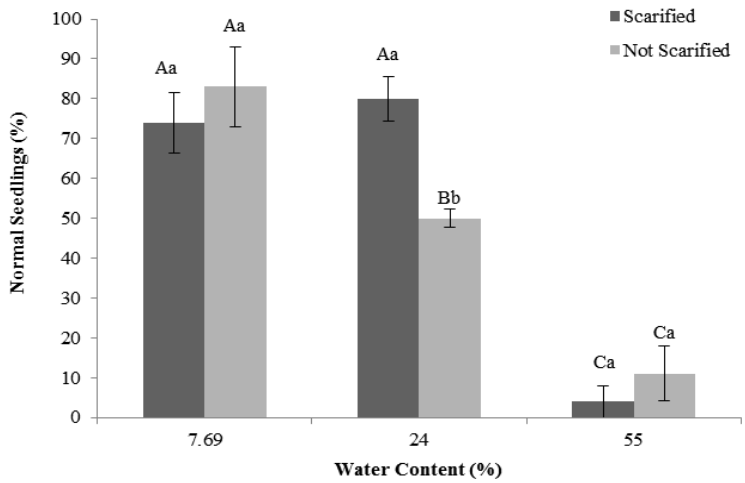

Figure 4. Desiccation tolerance in *Copaifera langsdorffii* seeds under the influence of the pre-germinative treatment. Same uppercase letters indicate the absence of differences between the water contents in a pre-germinative treatment by the Tukey test at 5% probability. Same lowercase letters indicate the absence of differences in the water content between pre-germinative treatments by the Tukey test at 5% probability.

For most species that produce orthodox seeds, the loss of desiccation tolerance always starts around the radicle protrusion, as observed for *Medicago truncatula* (Faria et al., 2005), *Sesbania virgata* (Masetto *et al.*, 2008), *Peltophorum dubium* (Guimarães *et al.*, 2011), *Solanum lycopersicum* and *Abelmoschus esculentus* (Lin et al., 1998). In all cases, sensibility happens only after the radicle protrusion. *Copaifera langsdorffii* is the first species reported with a premature loss of desiccation tolerance described in seeds from the Cerrado of Lavras (Pereira *et al.*, 2014). All provenances studied here and the first report for the species show that the loss of desiccation tolerance *by C. langsdorffii* starts at phase 1 and is completely lost in the middle of imbibition phase 2. Only *Senna multijuga* has been reported with a premature loss of desiccation tolerance (Rodrigues-Junior et al, 2014). This species has shown a loss of desiccation tolerance in the beginning of imbibition phase 2, and it was

completely lost in the middle of the same phase, whose behavior was similar to that presented by *C. langsdorffii*.

The effects of environmental characteristics on seed morphophysiology are already reported for many species (Wulff, 1986; Fenner, 1991; Gutterman, 2000). As an example, Bognounou *et al.* (2010) reported high effect of the environment under germination parameters of *Anogeissus leiocarpa, Combretum aculeatum, C. micranthum* and *C. nigricans.* For these species, germination and seedling establishment were influenced by seed provenance. Despite the premature loss of desiccation tolerance, *C. langsdorffii* seeds had an environmental-dependent behavior. Seeds from Montes Claros Cerrado could keep desiccation tolerance longer than the other two provenances (Figure 1).

Daws *et al.* (2006) reported a provenance-dependent level of desiccation sensitivity for *Acer pseudoplatanus*. The researchers showed that, for this species, a warmer condition results in a higher desiccation tolerance, when compared to seeds from cooler regions. The desiccation tolerance of *C. langsdorffii* shows a similar behavior, as can be observed in Figure 5. The region of Montes Claros has a warmer and dryer climate (Figure 5A) than Lavras. Seeds of *C. langsdorffii* develop from January to July, when dispersion, which happens until August, starts (Freitas & Oliveira, 2002; Lorenzi, 2002; Pedroni *et al.*, 2002). In Montes Claros, the rain season ends in May, and the seeds go through a dryer period during their development. Dryer conditions and higher temperatures can be an important factor to induce *C. langsdorfii* seeds to a desiccation-tolerant condition, when compared to the region of Lavras, which have lower temperatures and also a higher rainfall.

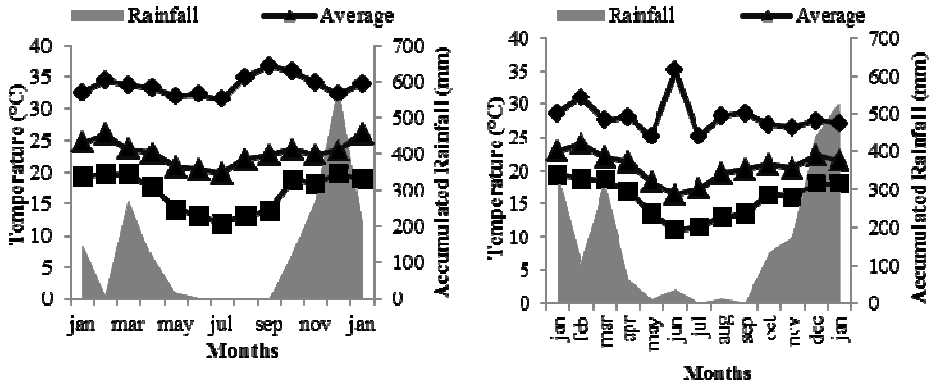

Figure 5. Climate data from Montes Claros (A) and Lavras (B). Source: A) Universidade Estadual de Montes Claros. B) Universidade Federal de Lavras.

Both samples from Lavras (Cerrado and riparian) had a similar behavior about the loss of desiccation tolerance, and was different from the sample of Montes Claros. Considering the differences in the climatic conditions between the regions, it is possible to conclude that the temperatures and rainfall are an important factor, inducing a higher desiccation tolerance in seeds. The importance of the climatic conditions on the seed desiccation tolerance cannot be separately considered. *Tapirira obtusa* seeds harvested in the same region (Lavras), but in different environments (Cerrado, riparian and rupestrian fields) show different behaviors, although developed in the same region. Thus, it is important to consider not only the climatic conditions, but also soil nutrients, water availability, as well as the presence and abundance of predators and species genetics (Pereira *et al.*, 2011).

The loss of desiccation tolerance in orthodox seeds as a function of maternal environment has not been yet described; however, many recalcitrant seeds have shown an environmental-dependent behavior regarding desiccation sensitivity. Seeds of *Camelia sinensis*, from South Africa, have been described as recalcitrant by Berjak *et al.* (1993), dying when dried at a water content below 29%; on the other hand, seeds from Japan survived to a 1°C storage by 6 years

and with a survival decrease of only 13% (Amma & Watanabe, 1985). Provenance has also influenced the average water content lost for that results on death of seeds of coffee (Ellis *et al.*, 1990).

C. langsdorffii is described with physical dormancy, causing the imbibition impediment of the seed (Fowler & Bianchetti, 2000). In the present study, it is possible to observe that the seeds do not have a uniform and slow germination when not submitted to mechanical scarification (Figures 3 and 4), which shows a delay in the germination of untreated seeds. Mechanical scarification is usually applied as a pre-germinative treatment for physically dormant seeds (Fowler & Bianchetti, 2000); however, for *C. langsdorffii*, it is not essential, since non-scarified seeds germinate, but without the uniformity presented in scarified ones.

Scarification allows a fast seed imbibition, which resulted in a uniform germination, but can also cause, as cited before, damage to the cell structure that could be a factor which influences the loss of desiccation tolerance in this species. In this case, a pre-humidification prior to imbibition was thought as a strategy to avoid the premature loss of desiccation tolerance (Crowe *et al.*, 1989; Kovach & Bradford, 1992). However, this treatment did not change the loss of desiccation tolerance during imbibition for *C. langsdorffii*, and the process happens with any expressive changes, when compared with seeds untreated before imbibition. There was no influence of mechanical scarification on desiccation tolerance. Non-scarified seeds showed a decrease in germination, when submitted to desiccation treatments, when compared to the scarified ones. It was possible to observe a decrease in desiccation tolerance at a water content of 24% for non-scarified seeds, which did not happen in scarified seeds, and, at this point, they are still desiccation-tolerant. Seeds with a water content of 55% had the lowest germination ratio, independent of scarification, showing that

mechanical scarification is not a factor that influences seed desiccation tolerance (Figure 4).

Conclusions

There is an influence of maternal environment conditions on the loss of desiccation tolerance of *Copaifera langsdorffii* seeds, and seeds from drier and warmer environments are more desiccation-tolerant than others.

Pre-germinative treatments do not influence the loss of desiccation tolerance.

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(VERSÃO PRELIMINAR)

ARTIGO 3 Validation of reference genes for *Copaifera langsdorffii* seeds during germination and desiccation tolerance re-establishment

VERSÃO PRELIMINAR submetida à revista *Brazilian Journal of Biology*, podendo sofrer alterações de acordo com o corpo editorial da revista.

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Running title: Reference genes for *Copaifera langsdorffii*

Abstract: Recently, the study of gene expression has been improving in technology and methods. For an accurate result, it is important to have a good set of reference genes. However, it is necessary to find this set for each species and study conditions. The present study has the objective to validate a good set of reference genes for germination studies on *Copaifera langsdorffii* seeds. Seeds with three imbibition times plus a desiccation tolerance re-establishment treatment were used in the experiments. Primers were designed using Fabaceae species sequences and tested in conventional PCR amplification, as well as real-time PCR efficiency. The remaining primers were tested on stability, and the analysis was performed using the tool GeNorm in the software qBase Plus and the Normfinder, using the software R for windows. The results of both algorithms were tested using a target gene, and the results of both GeNorm and

Norfinder were compared. In the conditions of the present study, the most stable genes were GADPH and AIN.
Keywords: Copaiba, germination, gene expression, housekeeping genes.

RESUMO: Atualmente, o estudo da expressão gênica tem melhorado em tecnologia e métodos. Contudo, para uma melhor precisão dos resultados é necessário se possuir um bom conjunto de genes de referência, e para isso é necessário o estudo para cada espécie e condição. Este trabalho teve como objetivo validar genes de referência comumente usados em espécies da família Fabaceae para estudos de expressão gênica e reestabelecimento da tolerância à dessecação em sementes de *Copaifera langsdorffii*. Sementes em três estágios de embebição e também submetidas a um tratamento de reestabelecimento da tolerância à dessecação foram utilizadas para o estudo. Os primers foram desenhados baseando-se em sequencias de espécies da família Fabaceae e testados em PCR convencional para teste de amplificação e também em PCR em tempo real para eficiência. Primers com eficiência apropriada foram testados em PCR em tempo real, utilizando-se todos os tratamentos escolhidos. Os resultados foram comparados pela ferramenta GeNorm e também pela ferramenta Normfinder. Os resultados indicaram que, para as condições estudadas, os genes GADPH e AIN são os mais estáveis em *C. langsdorffii*.
Palavras-chave. Copaíba, germinação, expressão gênica.

Introduction

Copaifera langsdorffii is one of the most important species of the genus *Copaifera* (Guerra *et al.*, 2006). In Brazil, *C. langsdorffii* can be found in the Northeast (Bahia), Center-west (Goiás and Mato Grosso do Sul) and the Southeast (Minas Gerais and São Paulo), growing in Caatinga, Cerrado and riparian forests (Queiroz *et al.*, 2014). As members of the genus, from the wood

of *C. langsdorffii*, an oil with medicinal interest is extracted (Freire *et al.*, 2006). The wood can be used for buildings, furniture, doors and floors (Lorenzi, 2002).

Gene expression studies are an important tool for understanding physiological processes, and have been increasing recently. Real-time PCR (qPCR) became the most used procedure for the quantification of gene expression levels (Chen *et al.*, 2010; Cordoba *et al.*, 2011). However, many errors can happen during RNA isolation and purification, the synthesis of cDNA and even during the qPCR reaction, resulting in erroneous results (Vandesompele *et al.*, 2002). Therefore, many strategies are used, but the best one is the use of housekeeping genes (reference genes). In theory, these genes present a stable expression level (with a small variation), irrespective of treatments, tissues or developmental stages studied (Vandesompele *et al.*, 2002). In general, the most used genes are those related to basic cell processes, such as ribosomal proteins, actin, tubulin, ubiquitin and the elongation factor (Czechowski *et al.*, 2005; Dekkers *et al.*, 2012).

Since the usual reference genes were assumed as stable, they were not validated as stable before the beginning of the research. However, recent studies have shown that these genes will not be always stable (Vandesompele *et al.*, 2002; Czechowski *et al.*, 2005), and there is not a universal reference gene that can be used in all studies (Die *et al.*, 2010; Dekkers *et al.*, 2012). Thus, it is important to validate reference genes for each study, and a minimal number of three genes is necessary. Therefore, many tools have been created, such as GeNorm (Vandesompele *et al.*, 2002) and Normfinder (Andersen *et al.*, 2004). Both compare expression levels among treatments and indicate the best reference genes for the study.

A large number of studies have been conducted with the objective to validate reference genes for many agronomical and model species, such as *Arabidopsis thaliana, Glycine max, Medicago truncatula, Vicia faba, Pisum*

sativun (Czechowski *et al.*, 2005; Jian *et al.*, 2008; Hu *et al.*, 2009; Gutierrez *et al.*, 2010) and many others, as cited by Die *et al.* (2010). On the other hand, these studies show that, although some genes are stable in several species and conditions, it is still necessary to validate their use before the beginning of the study.

Copaifera langsdorffii seeds have shown an uncommon behavior about desiccation tolerance (Pereira *et al.*, 2014), and the study of the gene expression in *C. langsdorffii* is important for understanding desiccation tolerance mechanisms; however, there are not good reference genes validated for the species. Also, since a good set of reference genes are validated in a certain condition, they are useful candidates for others. Thus, this study aimed to validate a good set of reference genes during the germination and desiccation tolerance re-establishment of *Copaifera langsdorffii* seeds.

Methods

Seed Collection

Seeds of *C. langsdorffii* were collected in a Cerrado area in Montes Claros, Brazil, in August 2011. Only seeds from mature (open) fruits were collected and had the aryl removed manually. The seeds were then dried to 10±2% in a climate-controlled chamber [20 °C, 60% relative air humidity (RH)]. The seeds were stored in a cold chamber at 5±2°C and 40±2% air humidity. For this study, three imbibition points were chosen: 0 (dry seeds), 72 and 120 hours. For imbibition, the seeds were mechanically scarified using sandpaper, as used by Pereira et al (2014). The seeds were subsequently conditioned in paper at 25°C and constant light. For the re-establishment of desiccation tolerance, the treatment was stablished based on previous experiments (data not published), being used the treatment of incubation of 96 hours imbibed seeds conditioned on 1µM Abscisic Acid solution for 72 hours at 15°C in the dark.

RNA isolation

For RNA isolation, 20 seed embryos that were frozen in liquid nitrogen were used, and were then extracted from the seeds. The frozen material was kept in deep freezer at -80°C until their use.

The seeds were grinded in a Mo Bio 96 Well Plate Shaker/MM 400, and the RNA was then isolated using a modified Hot Borat protocol (Wan & Wilkins, 1994). RNA integrity was evaluated by electrophoresis in agarose gel and the concentration was measured in Nanodrop. cDNA was synthetized from 1µg RNA, using the kit IScript (Bio Rad, Hercules, CA, USA) with the manufacturer protocol. cDNA was diluted 10 times and stored in freezer at -20°C.

Reference Gene selection and primer design

Reference genes were selected after a literature search and, for primer design, only sequences of mRNA coding region were selected. At least 3 sequences of species from the Fabaceae family (except those genes that were found in *C. officinalis* sequences) were used for primer design. The sequences were collected on the open database National Center for Biotechnology Information (NCBI,http://www.ncbi.nlm.nih.gov). Table 1 shows the species used for primer design.

For sequence design, they were aligned using the *software* BioEdit. From the alignment, regions with the highest conservation between the species were selected. Specific primers (0-fold degeneration) were designed, using the conserved regions of the gene sequences. The primers were then tested on their specificity, using the online tool Basic Local Alignment Search Tool (BLAST), also the NCBI website. Only primers with homology to the candidate genes were used for the analysis.

Table 1. Reference candidate genes, species used and number of primer designed for tests

Gene	Primers Designed (code)	Used Species
Actin 1	ACT	*Medicago truncatula; Caragana korshinskii; Cicer arietinum;*
Actin 2	ACT	*Medicago truncatula; Medicago sativa; Glycyrrhiza uralensis;*
Actin Depolymerizing Factor	ACDTF	*Medicago truncatula; Cicer arietinum; Glycine max; Lotus japonicus;*
Auxin Induced Nodulin	AIN	*Copaifera officinalis*
Cyclophilin	CYP	*Viciafaba; Arachis diogoi; Glycine max; Arachis hypogaea; Cajanus cajan; Vigna radiata;*
Elongation Factor 1α	ELF	*Copaifera officinalis*
Glyceraldeyde-3-phosfatase-dehydrogenase	GADPH	*Medicago truncatula; Cicer arietinum; Pisum sativum; Glycine max;*
Initiation Factor 4A	IF	*Medicago truncatula; Cicer arietinum; Glycine max; Lotus japonicus;*
Protein Phosphatase 2A	PP2A	*Medicago truncatula; Cicer arietinum; Glycine max;*
Ribossomal Protein 40S	40s	*Copaifera officinalis*
Ribossomal Protein 60S	60s	*Copaifera officinalis*
TIP 41	TIP4	*Caragana korshinskii; Cicer arietinum; Glycine max; Lotus japonicus;*
Ubiquitin	UBQ	*Cicer arietinum; Glycine max; Lotus japonicus; Medicago truncatula*

Primer amplification and efficiency tests

For primer amplification tests, a mix from all cDNA samples was used. The conventional PCR was then carried out, in order to check the amplification of each primer. PCR products were evaluated by agarose gel electrophoresis and only primers that had amplified were tested for their efficiency. The efficiency tests were performed by real-time PCR, using a mix of all samples for each primer with 3 replicates. Subsequently, the amplification data was used for the efficiency test. This test was made using the software LinReg, that showed the

average efficiency of each primer. Only primers that had efficiency between 1.9 and 2 proceeded for the stability test (Dekkers *et al.*, 2012).

Gene stability

For the stability test, a real-time PCR was carried out for each primer, using all samples individually at this time (3 biological and 2 technical replicates for each evaluated point). The threshold cycle (ct) data was used for the stability test, using the software qBase (Vandesompele *et al.*, 2002) and also the Normfinder package for the software R for Windows (Andersen *et al.*, 2004). By both softwares, an ideal set of reference genes should be those that present a stability average (m value) lower than 0.5, being acceptable at lower than 1. The data from both softwares were compared to select the best genes for *C. langsdorffii*.

Normalization factor comparisons

In order to make comparisons between the results, the expression profile of one target gene was done. In this case, the Superoxide Dismutase (SOD) gene was used. The primer designed presented amplification in conventional PCR and also presented efficiency (1.9). The expression level was normalized with the best three reference genes indicated by the softwares (with and withouth the re-establishment treatment) and was compared to a set of unstable genes.

Results and discussion

Figure 1 shows the gels from conventional PCR products of each designed primer in this study. From the 22 designed primers, only 15 showed specific amplification and were used for efficiency tests. The results of this second test are presented in Table 2. The average efficiency was between 1.6 (80%) to 2 (100%), but only the primers that presented an efficiency higher than 1.9 (95%) were checked for stability.

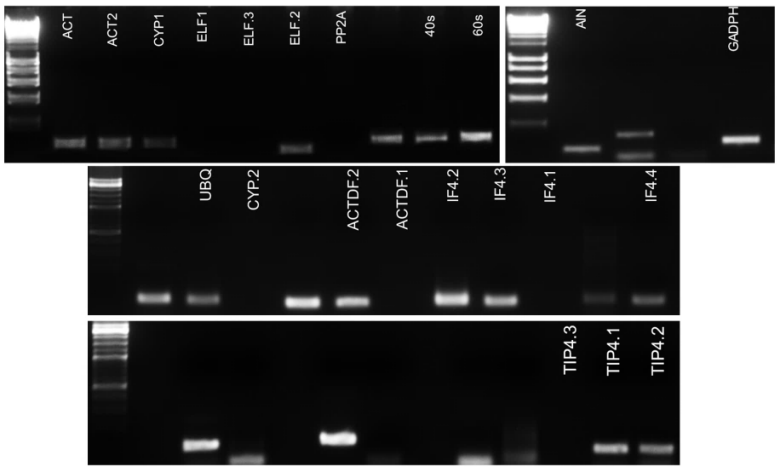


Figure 1. Agarose gels from PCR products of primers tested on *Copaifera langsdorffii*. Unidentified points are genes not used in the present study.

For the stability tests, data were analyzed using all treatments (0, 72 and 120 hours of imbibition plus the re-establishment treatment), and also without the re-establishment treatments. Figure 2 shows the GeNorm results for the stability of the data. When data were evaluated only for imbibition times (Figure 2A), it was observed that the genes 40s and GADPH were the most stable for the generation of the normalization factor. For an ideal normalization factor calculation, the use of a minimal of 3 reference genes is necessary (Vandesompele *et al.*, 2002). As mentioned by Cordoba *et al.* (2011), the use of two co-regulated genes, despite not influencing the stability test, is not recommended for use in normalization factor calculations. Thus, as 40s and 60s are ribosomal proteins, one of those must not be used. Therefore, the best genes for use are 40s, GADPH and AIN. When all treatments (imbibition times and the re-establishment treatments) were used, there was not a gene with stability lower than 1. The use of the three most stable genes for normalization factor calculations was indicated in the qBase software analysis. In this case, GADPH,

ACT1 and AIN are the genes that had stability lower than 1, when only imbibition treatments were evaluated.

Table 2. Average efficiency of the tested primers for *Copaifera langsdorffii*.

Gene	Primer Code	Efficiency Average
Actin 1	ACT1	1.91
Actin 2	ACT2	1.99
Actin depolymerizing factor	ACTDF.1	N/A*
	ACTDF.2	1.90
Auxin induced nodulin	AIN	2.00
Cyclophilin	CYP.1	1.60
	CYP.2	N/A
Elongation Factor 1α	ELF.1	N/A
	ELF.2	2.00
	ELF.3	N/A
Glyceraldeyde-3-phosfatase-dehydrogenase	GADPH	2.00
Initiation Factor 4A	IF.1	N/A
	IF.2	1.70
	IF.3	1.94
	IF.4	1.81
Protein Phosphatase 2A	PP2A	N/A
Ribossomal Protein 40s	40s	1.92
Ribossomal Protein 60s	60s	1.95
TIP41	TIP4.1	1.81
	TIP4.2	1.75
	TIP4.3	N/A
Ubiquitin	UBQ	1.68

*Primers without amplification in the conventional PCR. Only primers with an efficiency higher than 1.9 (95% efficiency) were used for the stability test.

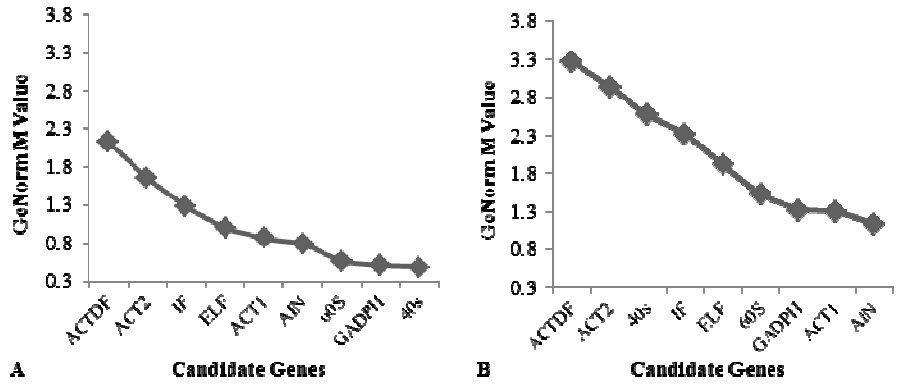


Figure 2. Average stability of the primers by the GeNorm analysis. **A.** Results for analysis with the treatments 0, 72 and 120 hours of imbibition. **B.** Results of analysis for 0, 72, 120 hours of imbibition and the treatment of desiccation tolerance re-establishment. The GeNorm M value was calculated with the GeNorm tool using the software qBase Plus®.

By the Normfinder analysis, the most stable genes are the same that were indicated by GeNorm. However, there are some differences between both analyses about the order of stability, as observed in Figure 3A. According to Normfinder, AIN is more stable than GADPH and 60s. When the re-establishment treatment was included, the most stable genes were AIN, GADPH and ACT1, as also observed by GeNorm (Figure 3B).

Both analysis (GeNorm and Normfinder), showed similar results about the stability of the genes. Differences between algorithms have been observed by Cordoba *et al.* (2011). However, in this study, despite the small differences between the analyses (about the stability order), the best three genes indicated for normalization were the same in all tests. That indicates 40s, GADPH and AIN as reference genes (for imbibition time analysis) and GADPH, AIN and ACT1, when the re-establishment treatment was included in this analysis.

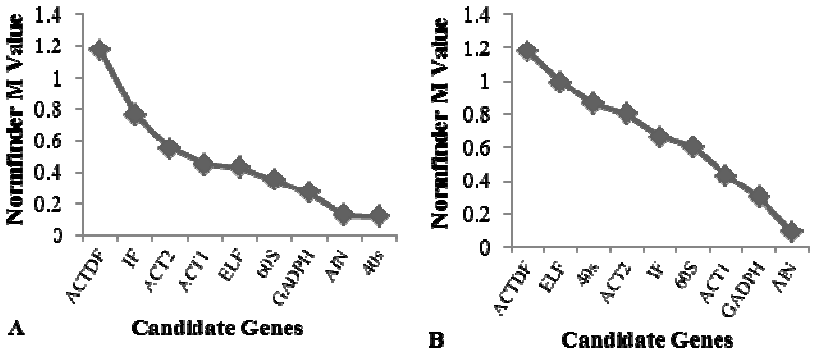
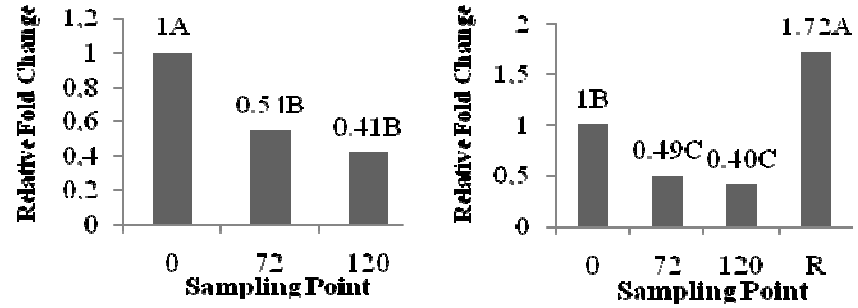


Figure 3. Average stability of the primers by the Norfinder analysis. **A.** Results for analysis with the treatments 0, 72 and 120 hours of imbibition. **B.** Results of analysis for 0, 72, 120 hours of imbibition after the re-establishment treatment of desiccation tolerance. The Normfinder M value was calculated with the package on the software R for Windows.

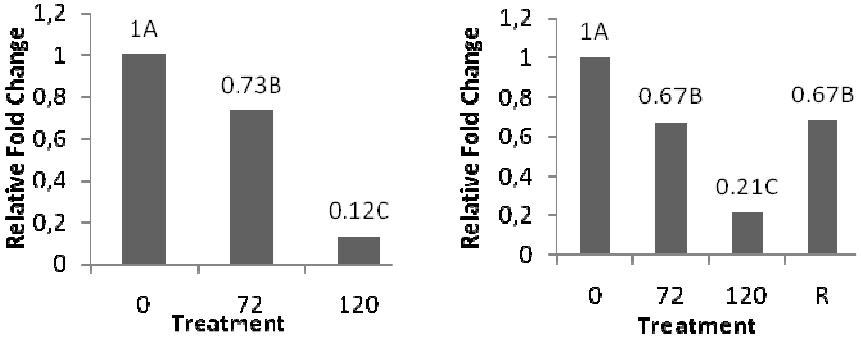
To proceed the gene test, a normalization test was carried out, using the target gene Superoxide Dismutase (SOD). For the tests without the reestablishment treatment, Initiation Factor 4A, Elongation Factor 1α and Actin depolymerizing factor were used. For the test with the treatment, Actin depolymerizing factor, Actin 2 and ribosomal 40s protein were used. The data from the target gene was also normalized using the best three reference genes. The result can be observed in Figure 4. Using the normalization factor generated by the most stable genes, the gene shows a down-regulated standard during imbibition, and has an increase when the seeds are submitted to the re-establishment treatment.

When the results without a good set of reference genes were analyzed the as standard can be observed in Figure 5), it was possible to observe that the gene expression standard has no change (down-regulated during imbibition and upregulated after the reestablishment treatment). However, there are some changes that can be observed when Figures 4 and 5 are compared.



A B

Figure 4. Normalized expression of SOD using the best three reference genes. **A.** When only imbibition times were analyzed. **B.** When imbibition times and the re-establishment treatments were analyzed.



A B

Figure 5. Normalized expression of SOD using three not validated reference genes. **A.** When only imbibition times were analyzed. **B.** When imbibition times and the re-establishment treatments were analyzed.

The arbitrary choice of a set of reference genes without a trustable validation has been mentioned as an uncertain way to study gene expression (Gutierrez et al, 2008). An unappropriated reference gene can generate an erroneous result and knowledge. The importance of the validation could be observed in the present study. The expression standard of the gene was down-

regulated during imbibition and, after the re-establishment treatment, it has increased up to a value higher than that of time 0 (Figure 4). When the expression was normalized using a bad set of reference genes, although the standard was the same, the values were different and not trustable. The major difference can be observed between the results after 72 hours of imbibition (Figures 4A and 5A) and after the re-establishment treatment (Figures 4B and 5B).

The study of reference genes has been mostly conducted for agronomical and model species such as *Arabidopsis thaliana* (Czechowski *et al.*, 2005; Dekkers *et al.*, 2012), *Glycine max* (Jian *et al.*, 2008; Hu *et al.*, 2009), *Brassica napus* (Chen *et al.*, 2010), *Zea mays* (Chen *et al.*, 2011), *Pisum sativum* (Die *et al.*, 2010). These data are the best starting point for reference gene research on another species.

There is not a universal set of reference genes which could be used for all species or conditions. For *Zea mays* and *Spinacia oleracea*, Chen *et al.* (2011) observed that GADPH is a good candidate. In the case of stress conditions for *Hedysarum coronarium*, Cordoba *et al.* (2011) indicate ubiquitin, while for *Pisum sativum*, Die *et al.* (2010) suggested Actin and Protein Phosphatase 2A. Actin 1 and Elongation Factor 1α were also cited for *Vicia faba* (Gutierrez et al., 2010). However, as showed by Hu *et al.* (2009), the set of reference genes depends on the conditions and tissue.

Reference genes can change according to the species and experimental conditions (Gutierrez *et al.*, 2008). GADPH and AIN were the best reference genes, once these were between the most stable genes under the conditions analyzed in the present study. Once the use of three reference genes is ideal, it is possible to choose between 40s and 60s (only for imbibition times) or ACT1 (when the re-establishment treatment is included). In the present study, good candidates as reference genes for *Copaifera langsdorffii* were found; however, it

is important to validate these genes for each study condition, since the expression of all of them can change.

Conclusions

Glyceraldehyde-3-phosphatase-dehydrogenase and Nodulin Induced Auxin can be used as reference genes for studies on *Copaifera langsdorffii* seed germination.

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(VERSÃO PRELIMINAR)

ARTIGO 4 Molecular aspects of the loss and re-establishment of desiccation tolerance in *Copaifera langsdorffii* seeds

VERSÃO PRELIMINAR submetida à revista *Annals of Botany*, podendo sofrer alterações de acordo com o corpo editorial da revista.

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Original Article

Running head title: Desiccation tolerance in *Copaifera langsdorffii* seeds

Abstract: *Copaifera langsdorffii* is an important species from the *Copaifera* genus. Its seeds, characterized as orthodox, show an uncommon behavior regarding desiccation tolerance, which is lost precociously during germination. Thus, this study was conducted with the objective to analyze changes in desiccation tolerance mechanisms during the germination of *C. langsdorffii* seeds. Therefore, changes in oligosaccharides, gene expression and protein profile of *C. langsdorffii* seeds were analyzed for the loss and re-establishment of desiccation tolerance. Raffinose family sugars are present only in non-imbibed seeds. From the total seed weight, 7.3% is composed by heat-stable proteins that decrease during the imbibition process. ZEP, HSP and SOD, GA 20-oxydase and CYP707A expression dynamics have shown a correlation with desiccation tolerance. In the present study, it was possible to observe a correlation of ZEP, HSP and SOD genes and the synthesis of heat-stable proteins with the loss of desiccation tolerance in *C. langsdorffii* seeds.

Keywords: Copaiba, desiccation sensitivity, gene expression, heat-stable proteins, oligosaccharides.

Introduction

Desiccation tolerance is defined as the ability of an organism to lose its water content until a critical level, keep on this state and reestablish the normal metabolism after rehydration (Alpert, 2000; Oliver *et al.*, 2000; Hoekstra *et al.*, 2001). Desiccation tolerance is present in all groups of organisms, from bacteria to animals. In plants, it is more common in pollen, spores and seeds. There are three seed classes, according to desiccation tolerance. Those highly tolerant, which are able to survive with a low water content and temperatures for a long time: they are called orthodox; in the opposite side, there are seeds sensitive to drying and storage, called recalcitrant (Roberts, 1973). Between the two groups, there are seeds that have an intermediate behavior, being classified as intermediate seeds (Ellis *et al.*, 1990).

A large group of plant species of interest (economical, ecological or medicinal) produces recalcitrant or intermediate seeds (Hong & Ellis, 1996; Bovi *et al.*, 2004). This is one of the major complications in seed technology, since these seeds cannot be stored for long periods. Due to this fact, desiccation tolerance is one of the major matters in seed biology; however, due to the problems with sensitive species, these ones are difficult to study, being the orthodox seeds, which behave as recalcitrant during germination, a useful model to understand desiccation sensitivity (Sun *et al.*, 1999).

Desiccation tolerance has been well studied on model species, such as *Arabidopsis thaliana* and *Medicago truncatula* (Meurs *et al.*, 1992; Nambara *et al.*, 1995; Wehmeyer *et al.*, 1996; Wehmeyer & Vierling, 2000; Buitink *et al.*, 2003; Vu *et al.*, 2003; Faria *et al.*, 2005; Buitink *et al.*, 2006). There are also many studies related to desiccation tolerance in another species (Lin *et al.*, 1998;

Masetto *et al.*, 2008; Guimarães *et al.*, 2011). These studies report as a rule that the loss of desiccation tolerance occurs around the radicle protrusion. However, for *C. langsdorffii*, this loss occurs between phase 1 and the middle of phase 2 (Pereira *et al.*, 2014), and it is the first species described with a different behavior, when compared to orthodox seeds which have been already studied.

The drying process changes many cell conditions, such as the increase in pH, membrane integrity, as well as the physical and chemical composition (Nedeva & Nikolova, 1997). Drying with an active metabolism also makes the cell incapable of eliminating toxic metabolites, such as oxidant agents that cause deleterious reactions in the cell (Leprince *et al.*, 1990; Leprince & Hoekstra, 1998). Drying can result in mechanical damage to cell membranes by the increase in hardness (Hoekstra *et al.*, 2001). To avoid drying damages, the cell must have mechanisms to keep its structure and DNA integrity (Pammenter & Berjak, 1999). Therefore, during the maturation stage, the seed starts accumulating reserve molecules, such as *late embryogenesis abundant* (LEA) proteins and raffinose family oligosaccharides. These molecules keep the cell and DNA structure by replacing water, and avoid cell components from leaching (Koster & Leopold, 1988; Leprince, 1993).

During the drying process, the seeds are vulnerable to oxidant molecules, and antioxidant systems are important to keep seed survival (Finkel & Holbrook, 2000; Laloi *et al.*, 2004). The most common antioxidant enzyme is *superoxide dismutase* (SOD), which catalyzes reactive oxygen species (ROS) into O_2. There are also another mechanisms, such as catalase, glutathione reductase and peroxidases.

Most of the desiccation tolerance studies show that abscisic acid (ABA) is majorly linked to desiccation tolerance. Most of the mechanisms are responsible for ABA and its presence can also induce desiccation tolerance. However, many other genes can be related to desiccation tolerance. Angelovici

et al. (2010) report that, in *Arabidopsis thaliana*, 30% of the plant genome changes during seed drying. One particular tool to understand the mechanisms linked to desiccation tolerance consists in the treatment of the seed in a sensitive stage with combinations of ABA, polietilenglicol (PEG) and low temperatures. The re-establishment has been used in many species (Bruggink & Toorn, 1995; Vu *et al.*, 2003; Faria *et al.*, 2005; Bruggink *et al.*, 2007; Vieira, 2008), and the gene expression of sensitive seeds before and after the re-establishment of desiccation tolerance is a useful model to understand the mechanisms related to the process.

Many desiccation tolerance mechanisms have been described for model species, and changes in sugar composition and storage were analyzed, as well as the expression and loss of desiccation-tolerance related genes. However, *C. langsdorffii* seeds have presented an uncommon behavior, and it is interesting to understand what mechanisms are related to this loss at phase 1 of germination. Thus, the objective of this study was to evaluate changes in sugars, gene expression and heat-stable proteins in *Copaifera langsdorffii* seeds during imbibition and desiccation tolerance re-establishment process.

Material and Methods

C. langsdorffii seeds were harvested from mature (open) fruits in July 2011 in *stricto sensu* Cerrado of Montes Claros (North of the State of Minas Gerais, Brazil); the seeds were then manually cleaned, removing the aryl and drying into storing water content (10 ± 2% at wet basis) in a climate-controlled chamber [20 °C, 60% relative air humidity (RH)]. After drying, the seeds were stored in sealed semipermeable plastic bags in a cold chamber (5 ºC, 40% relative air humidity), at the Forestry Seed Laboratory at Universidade Federal de Lavras until the analyses. For the physiological tests, the conditions of paper roll at 25°C under constant light for imbibition/germination procedures were

standardized, and the seeds were also submitted to mechanical scarification, according to the methods applied for desiccation tolerance studies on the species (Pereira *et al.*, 2014). The seeds had the loss of desiccation tolerance characterized in previous experiments (data not published), based on the same methodology used by Pereira *et al.* (2014).

Based on the above, seeds with 96 hours of imbibition (desiccation-sensitive) were used for testing re-establishment. Therefore, the seeds were conditioned in three combinations between polyethyleneglycol (PEG), abscisic acid (ABA). The solutions were PEG 0MPa/ABA 0µM, PEG 0 MPa/ ABA 1 µM and PEG 1.7 MPa/ABA 0 µM. Each of these combinations was tested at 10, 15 and 20°C for 72 hours in the dark. Desiccation tolerance was tested by the drying of the seeds at 30% air relative humidity, until reaching the initial water content (before the beginning of imbibition). The seeds were kept dry for 72 hours, when they were submitted to pre-humidification at 100% air water content and 25°C under constant light; they were then conditioned under the germination conditions for testing. Desiccation tolerance was evaluated by the normal seedling percentage, corrected on the basis of the initial germination percentage presented by untreated seeds.

In order to analyze proteins, gene expression and oligosaccharides, two imbibition points were used, according to the loss of desiccation tolerance in previously tested seeds, being 72 hours (still desiccation-tolerant) and 120 hours (desiccation-sensitive). The best re-establishment treatment was also used for comparisons. Seeds without imbibition were used as a control (0-hour imbibition). For gene expression and protein quantity, embryonic axis were used for sampling; however, for oligosaccharides, re-establishment was not analyzed; however, they have been tested in embryonic axis and cotyledons.

For oligosaccharides, 20mg of lyophilized material was incubated for 15 minutes at 76°C in a 80% methanol solution with 40mg/L melezitose (internal

standard). Subsequently, the remaining methanol was evaporated in a SpeedVac for 2 hours. The pellet was resuspended in 1 mL miliQ water and centrifuged at 14,000 rpm for 3 minutes. The supernatant was diluted 10 times and filtered for the HPLC analysis (Dionex, Carbopac PA 1 column electrochemical detection). The concentrations of oligosaccharides were used for data analysis. The amounts of inositol, trehalose, glucose, sucrose, raffinose and stachyose were analyzed in the seeds.

RNA was isolated using a modified Hot Borate protocol (Wan & Wilkins, 1994). In the extraction buffer (0.2M sodium borate decahydratate, 30mM EGTA, 1% (w/v) SDS, 1% (w/v) Sodium deoxycholate), 1.76 mg DTT were added, as well as 52.8 PVP, incubated at 80°C for 5 minutes. The buffer was added to 100mg of grinded material and mixed in vortex; 4µ of Proteinase K solution (1.35 mg at 4.4µ water) were then added and mixed. The samples were incubated at 42°C for 15 minutes (and mixed at each 5 minutes), and then 64µL of 2M KCl were added and incubated on ice for 30 minutes. The samples were centrifuged at 13,200 rpm at 4°C for 20 minutes and the supernatant was transferred into a new tube, where 259 µL of 8M ice-cold LiCl were added and incubated on ice overnight in a cold room. The samples were then centrifuged at 13,200 rpm at 4°C for 20 minutes, and the supernatant was discharged. The pellet was washed in 750µL of 2M ice-cold LiCl and centrifuged at 13,200 rpm at 4°C. The pellet was resuspended in 100µL of DEPC MiliQ water and the concentration was measured.

The DNAse treatment was carried out with 10µg RNA by the addition of 10µL Promega® DNAse enzyme and 10µL DNAse buffer, and the samples were incubated at 37°C for 20 minutes. In this solution, 100µL of Phenol chloroform solution were added and mixed on vortex. In a Phaselock® (previously submitted to centrifugation for 30 seconds), the solution was added and centrifuged for 5 minutes, and the upper phase was transferred into a new

tube with 1/10 volume of Sodium Acetate and 2.5 times the volume of ice-cold ethanol. The material was led to precipitate for 1 hour at -20°C and centrifuged at 13,200 rpm for 20 minutes at 4°C; the pellet was then washed in 250µL ice-cold ethanol. The pellet was dried at room temperature and dissolved in 20µL of DEPC MiliQ water. The integrity of RNA was evaluated by electrophoresis in agarose gel and the concentration was measured in Nanodrop. cDNA was synthetized from 1µg RNA, using the kit IScript (Bio Rad, Hercules, CA, USA), using the manufacturer protocol. cDNA was diluted 10 times and stored in a freezer at -20°C.

Genes related to seed metabolism and desiccation tolerance were selected, based on the literature. For primer design, complete sequences of mRNA were searched on the data base National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). For each gene, at least three sequences of Fabaceae family species were selected to the design (Table 1). The sequences were aligned using the software BioEdit and a highly conserved region with 600 to 1200 base pairs was used. Specific primers based on these sequences were designed for the analysis.

For primer amplification tests, a mix of all samples was used. A conventional PCR was then carried out to check the amplification of each primer. PCR products were evaluated by agarose gel electrophoresis and only primers that amplified were tested for their efficiency. The efficiency tests were made using a real-time PCR with a mix of all samples for each primer with 3 replicates. Subsequeuntly, the amplification data was used for the efficiency test. This test was made using the software LinReg PCR, that showed the average amplification efficiency of each primer. Only primers that had an efficiency between 95 and 100% proceeded for gene expression analysis tests (Dekkers *et al.*, 2012). Actin 1, Glyceraldeyde-3-phosfatase-dehydrogenase and Auxin

Induced Nodulin were used as internal controls (reference genes), based on previous tests (data not published).

Table 1. Species selected as a basis to primer design for *Copaifera langsdorffii* gene expression studies.

Gene	**Used Species**
Zeaxantin Epoxidase 1 (ZEP 1)	*Medicago truncatula, Glycine max, Cicer arietinum,*
ABA 8'Hydroxylase (CYP707A)	*Medicago truncatula, Glycine max, Phaseolus vulgaris, Cicerarietinum*
Aba Insensitive 3 (ABI3)	*Pisum sativum, Populustrichocarpa, Rosa canina*
Small Heat Shock Protein (HSP)	*Copaifera officinalis*
Mitochondrial Heat Shock Protein (mHSP)	*Copaifera officinalis*
Dehydration responsive element binding protein (DREB)	*Eremospartonson goricum, Galega orientalis, Sophora davidii*
Gibberellin 20 oxydase (GA20ox)	*Medicago truncatula, Glycine max, Phaseolus vulgaris, Pisum sativum*
Mmannosyl-oligosaccharide 1,2-alpha-mannosidase (MAN1)	*Medicago truncatula, Glycine max, Cicer arietinum*
Superoxide Dismutase (SOD)	*Medicago truncatula, Glycine max, Lotus japonicus, Cicer arietinum*
Gibberellin Regulated Protein 4 (MTR_7g010580) (GRP)	*Medicago truncatula, Glycine max, Lotus japonicus, Cicer arietinum*

The amounts of total and heat-resistant proteins were also analyzed. For the extraction, 100 mg of the grinded samples were added to 1mL of the extraction buffer (500mM TrisHCl pH 7.5, 5mM NaCl, 5mM $MgCl_2$, 0.001M protease inhibitor added with 1µL β-mercaptoethanol). The samples were mixed on vortex and centrifuged at 13,200rpm for 30 minutes at 4°C. The supernatant

was divided into two aliquots, one for the analysis of total proteins and other for heat-resistance. Heat-resistant aliquots were heated at 85°C for 15 minutes. Subsequently, both aliquots (total and heat-resistant) were centrifuged at 13,200 rpm for 30 minutes at 4°C. The supernatant was transferred into new tubes and the samples were quantified according to the method of Bradford.

The electrophoresis was carried out using acrylamide gel. Thus, 20µL of the loading buffer was added to the samples and heated at 95°C for 5 minutes, then applied on the gel. The gel was prepared with two parts, the first gel was prepared with 12.6% acrylamide (8 mL distilled water; 12.45 mL of 1.5M Tris-HCl with 0.4% SDS; 9.3 mL of 40% acrylamide solution; 150µL of 10% persulfate solution and 24µL TEMED), and the second part with 6% acrylamide (6.4 mL distilled water; 2.5 mL of 1.5M Tris-HCl with 0.4% SDS; 1.57 mL of 40% acrylamide solution; 30µL of 10% persulfate solution and 22.5µL TEMED). The electrophoresis was carried out at 160v for 7 hours at 15°C. The gel was fixed for 30 minutes with a fixation solution (40% methanol, 7% acetic acid), and colored for 2 days in a solution with 0.08% (w/v) Colloidal Coomasie Blue G-250, 1.6% (v/v) orthophosphoric acid and 12% (w/v) ammonium sulfate. The gel was subsequently discolored for 5 minutes in 0.26% (w/v) Trizma Base with pH adjusted to 6.5 with ortophosphoric acid and washed in 40% (v/v) methanol solution. The gel was kept in ultrapure water for 2 days and the image was then obtained with a high-resolution scanner. The image was analyzed with the software GelAnalyzer, and the bands were compared by differences in intensity.

For the gene expression analysis, the software qBase Plus® was used for normalization and expression data calculations. All data were analyzed using the software R for Windows (3.1.0) by the Tukey test at 5% probability, when differences between the samples were observed by ANOVA. For all molecular

assays results, the data on Relative Fold Change was transformed, based on the control (0 hours of imbibition), for comparison.

Results and discussion

A germination test carried out together with re-establishment experiments shows 93% seed germination; based on this percentage, the success of each re-establishment treatment was calculated. All treatments showed a small percentage of re-establishment, except for PEG 0 MPa and ABA 0 µM at 10°C, which have no desiccation tolerance. Three treatments showed a re-establishment higher than 50% (Figure 1). These treatments were not statistically different. However, for gene expression and proteomics studies, PEG 0 MPa was used with ABA 1 µM at 15°C, since they presented the highest normal seedling percentage.

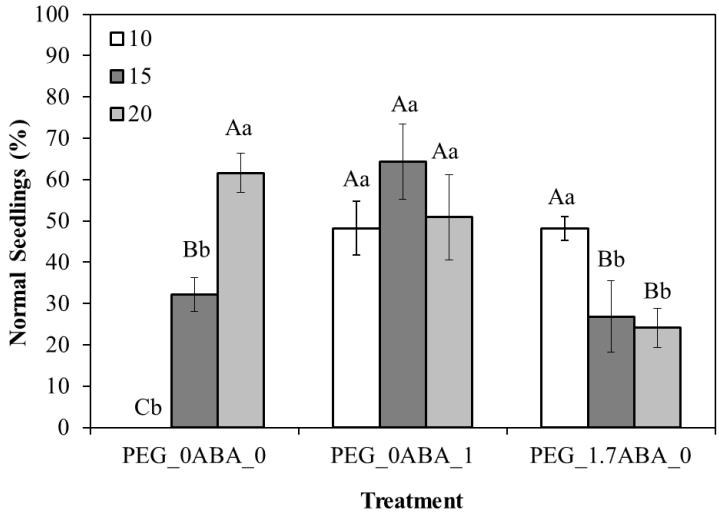

Figure 1. Normal seedling formation of *Copaifera langsdorffii* under desiccation tolerance re-establishment treatments. Same uppercase letters indicate the absence of the effect of temperature on the treatment. Same lowercase letters indicate the absence of the effect of the treatment on temperature.

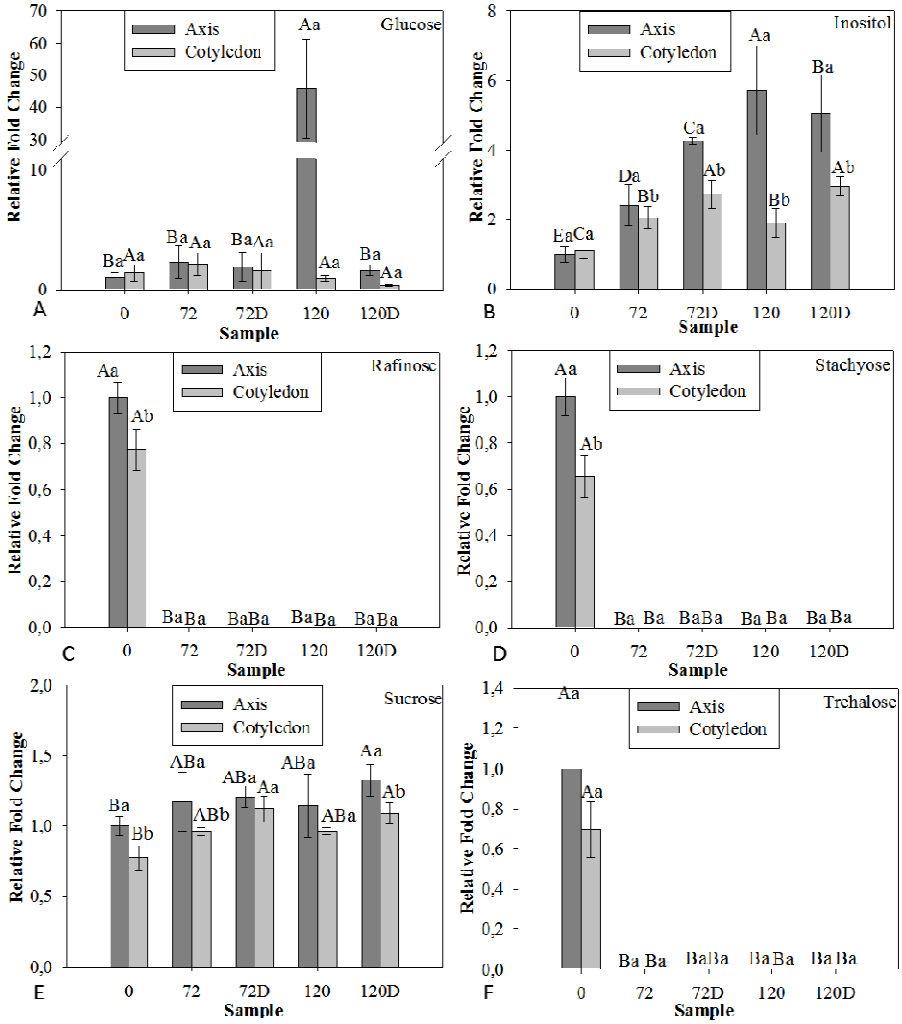

Figure 2. Changes in oligosaccharides of *Copaifera langsdorffii* seeds during imbibition and drying conditions. A) Glucose, B) Inositol, C) Raffinose, D) Stachyose, E) Sucrose, F) Trehalose. Data were normalized in Relative Fold Change, based on the concentration of the oligosaccharide in axis at 0 hours of imbibition. Same uppercase letters show the absence of differences in the oligosaccharide between the evaluated samples. Same lowercase letters show the absence of differences in the oligosaccharide between tissues. Sample captions: 0, 72 and 120 refer to the three imbibition points analyzed, 72D and 120D refer to samples with 72 and 120 hours imbibition (respectively), followed by drying, until reaching the initial water content.

The levels of oligosaccharides showed a variation between the samples. The major increase was in glucose on Axis at 120 hours imbibition (Figure 2A). There was an increase of 45 times (related to axis at 0 hours). At this point, seeds were in the middle of imbibition phase 2, and the germination was between 144 and 168 hours. Glucose levels kept without big changes, except for 120 hours; there was no difference about the levels of glucose between axis and cotyledon. At 120 hours, followed by drying, glucose levels decreased in the axis.

Inositol levels increased in axis during imbibition, even at 72 hours followed by drying, there was an increase in these levels. Only at 120 hours followed by drying, the levels of inositol decreased. These levels also increased in cotyledons; however, they decreased at 120 hours (Figure 2B). No big differences were observed in sucrose levels, except the fact that, at 120 hours, imbibed seeds followed by drying had more of this oligosaccharide than at 0 hours, in cotyledons and axis (Figure 2E). Raffinose, stachyose and trehalose (Figures 2C, D and F) showed the same standard. These oligosaccharides were only found in seeds without imbibition (0 hours), being absent in another sampling points.

During imbibition, as well as for re-establishment, there were changes in the expression of all analyzed genes. ZEP1 shows as a decrease during imbibition and, at 120 hours, the lowest expression levels of the gene were observed (Figure 3A); the expression restarted at the re-establishment treatment. The same standard was observed for both heat-shock proteins (Figures 3E and F), the expression levels in the re-establishment was lower than that observed in non-imbibed seeds. Since ZEP 1 expression decreased, ABA 8’hydroxylase increases (Figure 3B); however, even in the re-establishment treatment, the levels of this gene keep increasing. The same could be observed for Gibberellin

20-Oxydase and Gibberellin Responsible Protein, which increase during germination and keep this way during the re-establishment (Figures 3C and D).

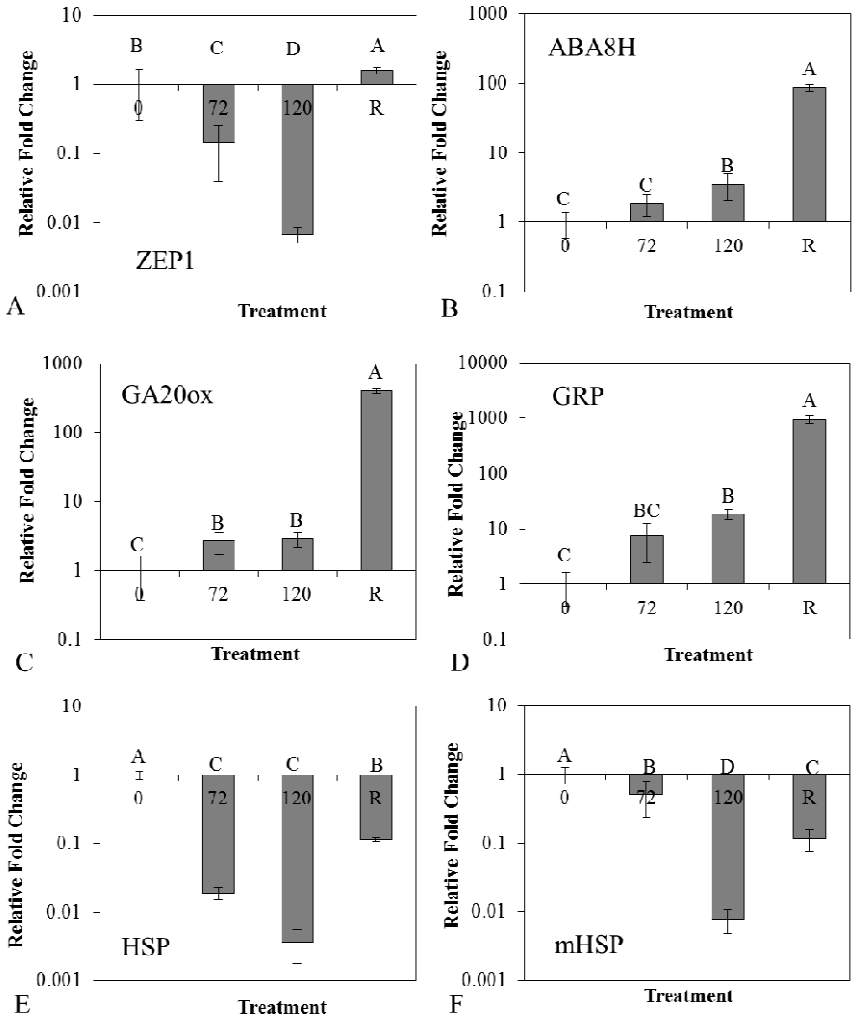

Figure 3. Changes in gene expression of *Copaifera langsdorffii* seeds during imbibition and desiccation tolerance re-establishment. A) Zeaxantin epoxidase 1, B) ABA 8' hydroxylase, C) Gibberellin 20-oxidase, D) Gibberellin Responsible Protein 4, E) Heat Shock protein, F) Mitochondrial Heat Shock Protein.

The gene ABI3 showed a decrease during germination and did not increase after re-establishment (Figure 4A). For MAN1 and DREB, the expression keeps increasing, even after the re-establishment treatment (Figures 4B and C). Superoxide dismutase decreased during imbibition, with the lowest levels at 120 hours. After the re-establishment treatment, the levels increased in a higher rate, when compared to non-imbibed seeds.

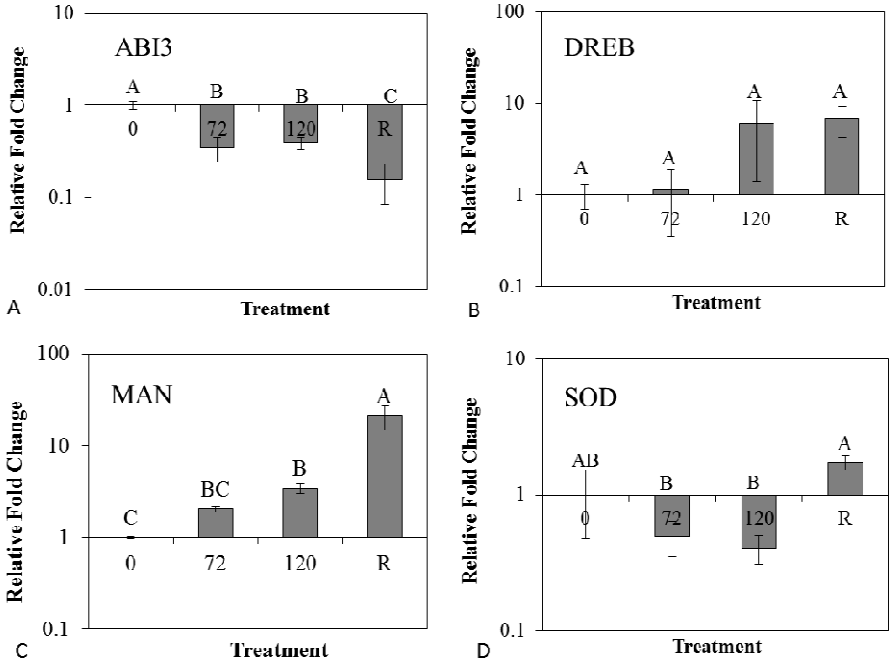

Figure 4. Changes in gene expression of *Copaifera langsdorffii* seeds during imbibition and desiccation tolerance re-establishment. A) ABA insensitive 3, B) Dehydration responsive element binding protein (DREB), C) Mannosyl-oligosaccharide 1,2-alpha-mannosidase (MAN1), D) Superoxide Dismutase 1 (SOD).

The initial percentage of total proteins in *C. langsdorffii* seeds was 8.71% (w/w). This amount decreased at 120 hours, being increased again at re-establishment (Figure 5). For heat-resistant proteins, the initial percentage was

7.30% (w/w); this shows that a large amount of proteins in the seeds are heat-resistant; however, this percentage decreased during imbibition, being increased again after the re-establishment. Total proteins increased at 72 hours, and this is probably related to the restart of seed metabolism, while the seed starts to produce new proteins for the radicle protrusion which, in this *C. langsdorffii* sample, happens between 144 to 168 hours. The same happens at the re-establishment treatment, that is, the increasing can be explained by the increase in heat-resistant proteins. On the other hand, the decrease at 120 hours could be related to the decrease in heat-resistant proteins at this point.

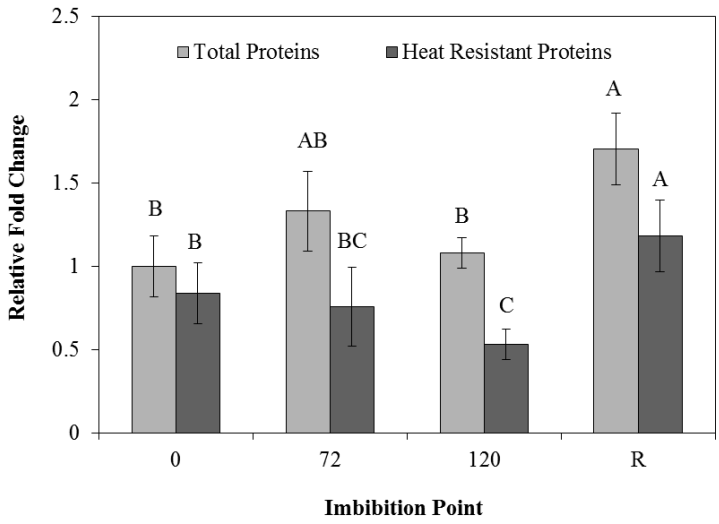

Figure 5. Changes in total and heat-resistant proteins of *Copaifera langsdorffii* seeds during imbibition and desiccation tolerance re-establishment. Data was normalized to Relative Fold Change, based on the percentage (w/w) of total proteins observed at 0 hours of imbibition. Same letters for a group of proteins indicate the absence of differences between the imbibition points.

From the gel analysis (Figure 6), it is possible to observe that, from all proteins isolated, 7 showed changes in expression levels between the sampling points. From these, 6 are heat-stable proteins and 1 was observed among heat-sensible proteins

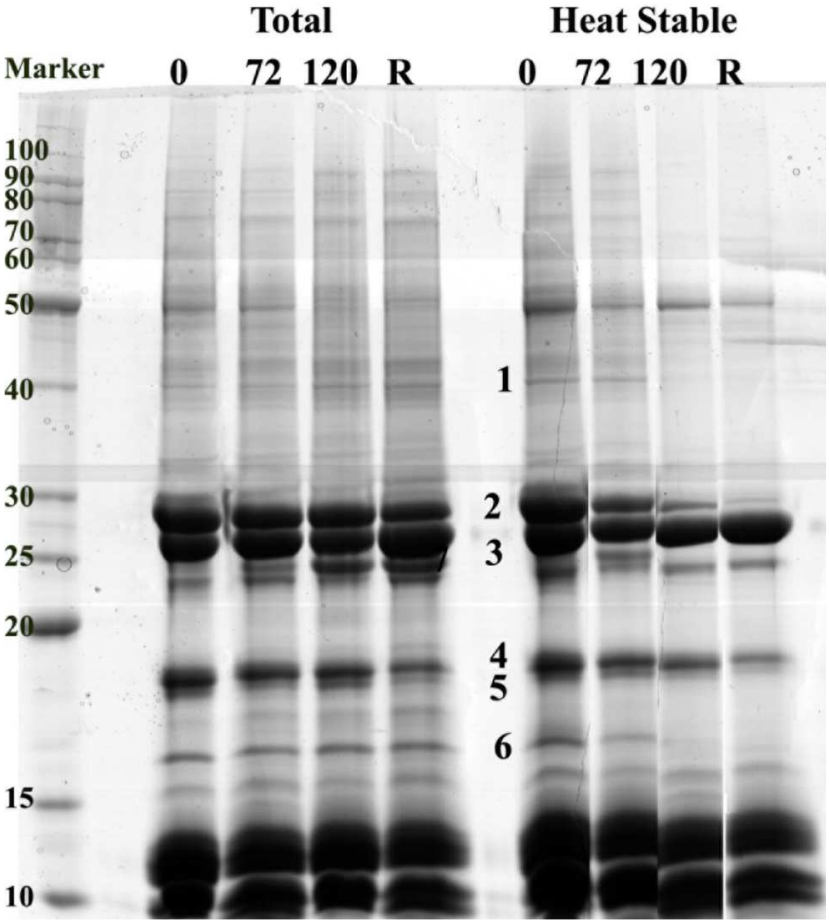

Figure 6. Total and heat-stable proteins of *Copaifera langsdorffii* seeds during imbibition and desiccation tolerance re-establishment treatments.

The data collected from the GelAnalyzer software showed differences in the seven bands selected (Figure 6). All heat-stable proteins showed a decrease in expression levels among the sampling points (Figures 6, 7A, B, D, Figures 8A

and B). However, protein 3 was expressed only at 72 hours, being absent in another points.

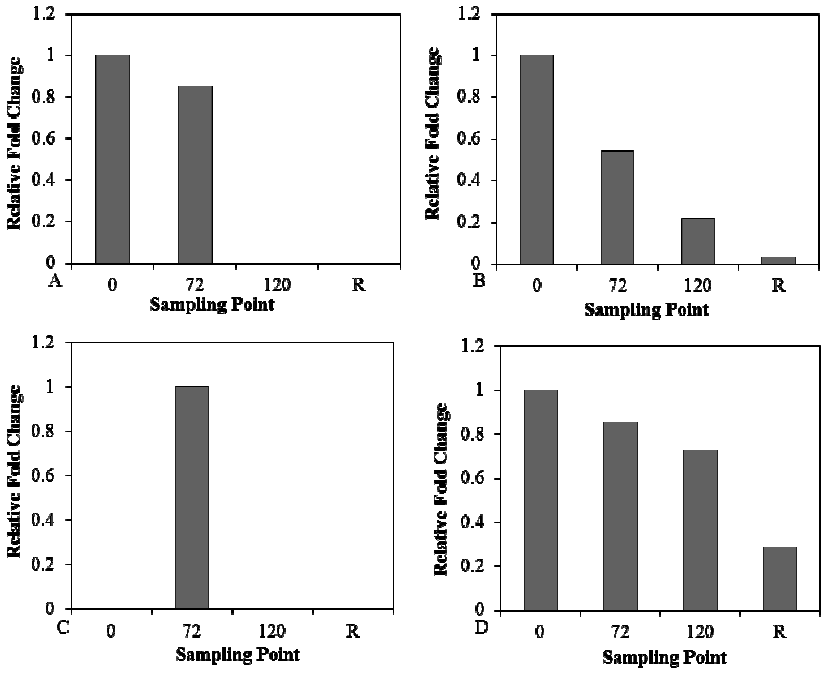

Figure 7. Relative changes in protein expression of *Copaifera langsdorffii* seeds during imbibition and re-establishment. (A) Protein 1, (B) Protein 2, (C) Protein 3, (D) Protein 4 (see Figure 6). Proteins 1, 2 and 4 had the relative fold change calculated based on band intensity at 0 hours imbibition. Protein 3 was calculated based at 72 hours.

Protein 7 showed an increase during the imbibition treatments, being absent at 0 hours and reaching the highest levels at 120 hours (Figure 8C). At the re-establishment point, this protein showed a decrease in expression.

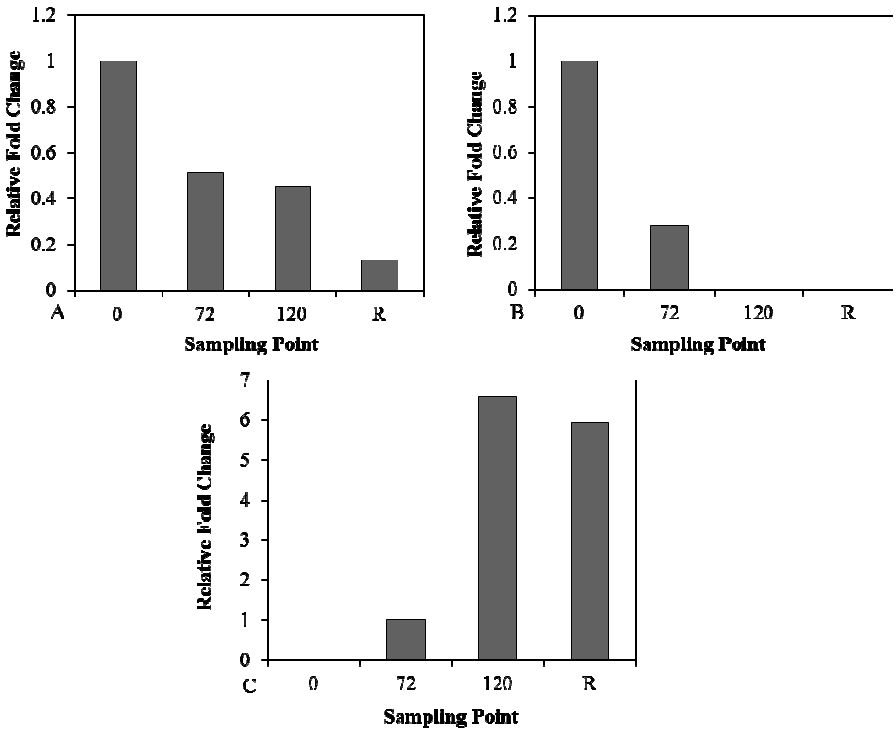

Figure 8. Relative changes in protein expression of *Copaifera langsdorffii* seeds during imbibition and desiccation tolerance re-establishment. (A) Protein 5, (B) Protein 6, (C) Protein 7 (see Figure 6). Proteins 1 and 2 had the relative fold change calculated based on band intensity at 0 hours imbibition. Protein 3 was calculated based at 72 hours.

Many mechanisms are required for the success of desiccation tolerance (Berjak, 2006). The accumulation of oligosaccharides, as an example, is important to protect the cell against the effects of the dying process. In *C. langsdorffii* seeds, the presence of raffinose family sugars (raffinose, stachyose and trehalose) were observed in non-imbibed seeds and the absence after the beginning of the germination (Figures 2 C, D and F). Sucrose and raffinose (trehalose, raffinose and stachyose) accumulation has been thought as involved to seed desiccation tolerance in many species (Blackman *et al.*, 1992; Blackig *et*

al., 1996; Black *et al.*, 1999), showing the importance of those sugars to protect the cell structure in the dry status for many researchers. These sugars and some proteins act replacing the water in the cell, and protects against the collapse of membranes and DNA (Koster & Leopold, 1988; Leprince, 1993; Black *et al.*, 1999; Pammenter & Berjak, 1999; Kranner & Birtic, 2005).

Myo-inositol is mentioned as an important factor for plant growth (Loewus & Murthy, 2000; Donahue *et al.*, 2010), playing a role in many cell processes. This oligosaccharide is synthetized from glucose and is a substract for raffinose family sugars (Shi *et al.*, 2005), being this synthesis reversible. It is already reported that these raffinose family oligosaccharides rapidly disappear after the beginning of imbibition, breaking down before the completion of polymeric carbohydrate mobilization (Blöchl *et al.*, 2007). Energy is highly required for the germinative process, as mentioned by Blöchl *et al.* (2007); the important role of raffinose family oligosaccharides in the energy supply of the early germinative process. As mentioned by Wang *et al.* (2003), these sugars are also antinutritional; therefore, the metabolism is necessary for the beginning of germination. Thus, the decrease in these sugars, rather than the increase in inositol and glucose, is probably related.

Black *et al.* (1999) reported an accumulation of raffinose together with the desiccation tolerance in *Triticum aestivum* embryos. For *C. langsdorffii*, these sugars are absent after the imbibition starts. However, at 72 hours, there is still a high desiccation tolerance (compared to 0 hours), but there is no raffinose family sugars. Thus, these sugars are not essential for the acquisition of desiccation tolerance, but can play an important role in this ability.

ABA is a major hormone in seed desiccation tolerance (Bewley & Black, 2013; Chandler & Robertson, 1994; Nambara *et al.*, 2000; De Castro &Hilhorst, 2006), in its metabolic pathway, Zeaxanthin epoxidase play an important role in the beginning of production (Cutler & Krochko, 1999). Most

desiccation tolerance mechanisms are responsible for the presence of ABA, which is rapidly synthetized under stress conditions (Zhang *et al.*, 2006). However, ABA must be degraded for the beginning of germination, as observed in this study and already reported. ABA 8'-hydroxylase (CYP707A) is known as one of the major enzymes in the ABA catabolism (Cutler & Krochko, 1999): its expression increases during germination, which is expected. The increase in the biosynthesis of GA is also important for the beginning of germination (Groot & Karssen, 1987; Debeaujon & Koornneef, 2000). Besides, the high expression of superoxide dismutase (SOD) in dry status and the re-increase in re-establishment show an important role of antioxidant mechanisms in desiccation tolerance (Pammenter & Berjak, 1999; Walters *et al.*, 2005).

In the present study, it was also possible to observe a decrease in the expression of ABI3 (Figure 4A), which also plays an important role in desiccation tolerance (Ooms *et al.*, 1993; Khandelwal *et al.*, 2010). However, this gene did not increase after the re-establishment treatment. Heat-shock proteins also showed a decrease during germination (Figures 3E and F); this could be confirmed by the protein analysis, once the heat-stable proteins (HSP class) decreased during germination. On the other hand, DREB 1 showed differences between imbibition times or after the re-establishment. This gene also plays a role in desiccation response, and a decrease during imbibition was expected, as well as an increase after the re-establishment treatment (Morran *et al.*, 2011). The decrease in superoxide dismutase was observed during the germination process.

During the imbibition/germination process, desiccation tolerance mechanisms are expected to decrease, while those ones related to germination must increase in order to restart the seed metabolism/germination (Angelovici *et al.*, 2010). In the present study, a reduction in desiccation tolerance mechanisms during germination was observed, as can be showed by changes in raffinose,

stachyose and trehalose (Figures 2C, D and F), desiccation-related genes, such as ZEP1, HSP and SOD (Figures 3A, E, F and 4D), decrease in heat-stable proteins (Figure 5) and increase in CYP707A, GA20ox and MAN1 (Figures 3B, C, 4C and D).

Considering the three phases in the imbibition/germination process, *C. langsdorffii* seeds start to lose their desiccation tolerance between imbibition phases 1 and 2 (Pereira *et al.*, 2014). As reported above, in most orthodox seeds, phase 1 is mostly characterized by water absorption, and most repairing and activation of the metabolic system are in the beginning of phase 2 (Bewley &*et al*, 2013). At this point, there is a high demand of energy, where oligosaccharides are useful (Blöchl *et al.*, 2007). Thus, the beginning of the loss of desiccation tolerance is expected between imbibition phases 2 and 3, when high metabolic events start (Bewley *et al*, 2013). However, *C. langsdorffii* loses its desiccation tolerance in phase 1, and sugars, gene expression and protein profiles change in this germination step. It suggests that, for *C. langsdorffii*, phase 1 is already characterized by high metabolic events which result in a premature loss of desiccation tolerance for this species.

The re-establishment in *C. langsdorffii* seeds showed a reasonable efficiency, since it was possible to have more than 60% of seed re-establishment (based on the initial germination percentage). When gene expression and proteins were analyzed after the re-establishment, it was possible to observe a new increase in the expression of ZEP, HSPs and SOD, which had decreased during the germinative process. The percentage of heat-stable proteins follows the same standard of these genes. However, germination-related genes (GA20ox, CYP707A and MAN1) did not decrease after the re-establishment, and there was also a decrease in ABI3, even after the re-establishment treatment, which suggests no relationship of these genes with desiccation tolerance. Despite the importance of oligosaccharides in the structural protection of the seed during the

dry status, HSPs and thermo stable proteins play the same structural role, which suggests that these proteins are replacing the functions of sugars that were consumed during germination.

Conclusion

The expression of Zeaxanthin epoxidase, Heat Shock Proteins and Superoxide dismutase genes, production of raffinose family sugars and also heat-stable proteins is correlated with desiccation tolerance in *Copaifera langsdorffii* seeds, present in desiccation-tolerant stages and absent in sensitive ones.

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