CIMILLE GABRIELLE CARDOSO ANTUNES

CARACTERIZAÇÃO FISIOLÓGICA E MOLECULAR DE SEMENTES DE *Poincianella pyramidalis* (TUL.) L. P. QUEIROZ (LEGUMINOSAE-CAESALPINIOIDEAE) SUBMETIDAS A ESTRESSES ABIÓTICOS

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Tese apresentada ao Programa de Pós-Graduação em Botânica, como parte dos requisitos exigidos para obtenção do título de DOUTORA EM BOTÂNICA, na área de concentração em Fisiologia Vegetal.

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FEIRA DE SANTANA, BA

DEZEMBRO, 2012

Aos meus queridos pais Edésio e Ana

e ao meu marido Daniel pelo apoio

incondicional desde sempre...

Dedico

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Augusto Cury

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SUMÁRIO

INTRODUÇÃO GERAL

A reprodução por sementes é uma característica particular das plantas superiores. Após o período de desenvolvimento (embriogênese e maturação), as sementes de caráter ortodoxo experimentam uma fase de dessecação, com uma progressiva redução das atividades metabólicas e síntese de compostos e proteínas protetoras que vão auxiliar na manutenção celular durante o estádio seco. Uma vez que as sementes sejam reidratadas, elas iniciam o processo de germinação que culmina com a protrusão radicular, reativando o metabolismo que se encontrava em níveis basais.

A formação de sementes de alta qualidade é pré-requisito básico para geração de plântulas vigorosas, que tolerem os diversos tipos de estresses bióticos (patógenos, por exemplo) e abióticos (seca, calor, salinidade, frio) que limitam o rendimento agrícola. Neste contexto, o uso de técnicas que possam aumentar o vigor de sementes como o pré-condicionamento, pode ter um efeito positivo na produção vegetal. O osmocondicionamento é uma técnica pós-colheita que pode proporcionar aumento da velocidade e uniformidade de germinação de sementes, especialmente sob condições adversas. A técnica preconiza o uso de agentes osmóticos (como o polietilenoglicol) para controlar a absorção de água nas sementes, aumentando o vigor das mesmas. Melhorias no vigor de sementes de uma série de espécies cultivadas têm sido reportadas na literatura.

Sabendo-se que o osmocondicionamento pode propiciar aumento do vigor e da tolerância a estresses abióticos em sementes, o estudo das bases fisiológicas (através do envelhecimento acelerado, da análise de crescimento de mudas, do armazenamento e respostas de tolerância cruzada) e moleculares (identificação de genes com expressão diferencial) que respondem a tais características em sementes não deve ser negligenciado.

Este trabalho objetivou investigar o comportamento germinativo das sementes da espécie *Poincianella pyramidalis* (nativa da Caatinga) em diferentes condições de temperatura e potenciais osmóticos, estabelecendo concomitantemente as melhores condições para o osmocondicionamento das sementes. Com isso, procurou-se identificar se o pré-condicionamento osmótico das sementes promove melhorias na germinação, na longevidade, no crescimento e desenvolvimento de plântulas e numa escala mais detalhada, quais genes poderiam ser responsivos ao osmocondicionamento aplicado às sementes.

REVISÃO DE LITERATURA

Caracterização da espécie em estudo

A família Leguminosae é a terceira maior família de plantas, ocorrendo em todos os tipos de ambientes terrestres e exibindo grande diversidade morfológica. Com distribuição cosmopolita, inclui 727 gêneros e 19327 espécies, dentre elas, a *Poincianella pyramidalis* (Tul.) L. P. Queiroz (QUEIROZ, 2009). Conhecida vulgarmente como catingueira é uma espécie endêmica do bioma Caatinga, com larga ocorrência no Nordeste Brasileiro. Sua distribuição vai desde o estado do Piauí até a Bahia (BRAGA, 1960; MAIA, 2004; FABRICANTE et al., 2009).

A catingueira apresenta uma ampla faixa de tolerância ambiental, exibindo um porte arbóreo quando a disponibilidade hídrica é elevada, e um porte arbustivo quando o suprimento hídrico é restrito. A espécie adapta-se facilmente a diferentes tipos de solos (desde os mais rasos aos mais profundos), com germinação rápida após as primeiras manifestações de chuva. Suas sementes (as quais apresentam comportamento ortodoxo de tolerância a dessecação) (ANTUNES et al., 2010) podem permanecer nos solos formando bancos, até que a umidade ideal desencadeie o início da germinação (BERNARDES, 1999; ARAÚJO, 2000; MAIA, 2004).

Sendo uma espécie de caatinga, a catingueira investe na caducifolia durante o período da seca, uma estratégia importante na economia de água, porém na estação chuvosa, ela é considerada uma anunciadora de chuvas, devido ao rápido rebrotamento das folhas em resposta ao aumento da umidade relativa. Tais folhas são bipinadas, de coloração rosada quando jovens e esverdeadas quando maduras. Ao alcançarem a maturidade ganham um cheiro desagradável característico da espécie. As flores são racimos curtos de coloração amarela. Os frutos são vagens achatadas de coloração castanho-claro quando maduros e as sementes também são achatadas de coloração castanho-claro quando maduras, medindo 1,2 x 0,9 cm de diâmetro em média. A casca exibe uma coloração de “camuflagem” amarelo, verde e branco e a raiz é pivotante (MAIA, 2004) (Figura 1).

P. pyramidalis é uma espécie considerada de potencial múltiplo, visto que toda a planta tem utilidade para a população sertaneja. Sua madeira é utilizada para obtenção de lenho, carvão, estacas, mourões e para produção de combustível (MAIA, 2004; SILVA et al., 2009); suas flores, folhas e cascas são utilizadas como expectorante, afrodisíaco e no

tratamento de bronquites, infecções respiratórias, influenza, asma, gastrite, cólicas, febre, diarréia, diabetes e dores estomacais (ALBUQUERQUE et al., 2007); por apresentarem alto valor protéico, as folhas constituem uma excelente alternativa de forragem durante a seca (MAIA, 2004); os ocos do caule servem como ninho para uma diversidade de abelhas, especialmente do gênero *Centris* (AGUIAR et al., 2003) e extratos das folhas apresentam atividade antioxidante (SILVA et al., 2011).

Figura 1. *Poincianella pyramidalis*. A) Vista geral da espécie; B) Fruto imaturo (Vagem); C) Flor (Racimo amarelo); D) Sementes de catingueira maduras.

Influência da temperatura e da deficiência hídrica na germinação

Estresses ambientais têm sido extensivamente estudados devido aos impactos causados no crescimento e desenvolvimento das plantas e produtividade das culturas (RODRIGUES et al., 2005). Alguns autores têm proposto que há uma correlação entre o aumento da frequência de eventos ambientais extremos e o aquecimento global, requerendo entre outras coisas, o estudo dos níveis de tolerância ambiental das espécies (PAPDI et al., 2009; DIAS et al., 2011).

Dentre os principais fatores abióticos capazes de interferir na germinação e no estabelecimento de plântulas, temperatura e disponibilidade hídrica parecem ter grande destaque no cenário agronômico, econômico e ecológico (PAPDI et al., 2009). A temperatura em que ocorre a germinação tem grande influência sobre esse processo, tanto no aspecto da porcentagem de germinação final como na velocidade de germinação, pois a mesma influencia na velocidade de absorção de água e na velocidade das reações bioquímicas (CARVALHO e NAKAGAWA, 2000; MARCOS FILHO, 2005).

Sementes de diferentes espécies apresentam faixas distintas de temperatura para germinação, as quais caracterizam sua distribuição geográfica e auxiliam em estudos ecofisiológicos (LIMA et al., 2011). A temperatura ótima de germinação é bastante variável entre as espécies, e a maior parte das espécies tropicais germinam numa faixa que vai de 20 a 30ºC (BORGES & RENA, 1993). Alguns trabalhos com leguminosas da caatinga corroboram os achados de Borges e Rena (1993) como, Guedes et al. (2010) que apontaram 30ºC como temperatura ótima de germinação da *Amburana cearensis*; José et al. (2011) que também indicaram 30ºC como melhor temperatura para germinação de *Apuleia leiocarpa* e Mondo et al. (2008) que encontraram 25ºC como temperatura ótima de germinação de *Parapiptadenia rigida*.

A disponibilidade hídrica é um outro fator limitante à germinação, especialmente para espécies nativas de regiões áridas e semi-áridas como *P. pyramidalis*, uma vez que o regime de chuvas é bastante irregular (ADAMS et al., 1999). A capacidade das sementes de algumas espécies em germinar sob condições de estresse hídrico confere vantagens ecológicas em relação a outras que são sensíveis à seca. Muitas pesquisas têm procurado determinar o potencial osmótico capaz de fazer cessar a absorção de água pela semente com o intuito de determinar níveis críticos de germinação (PEREZ et al., 2001; ROSA et al., 2005; CHAUHAN & JOHNSON, 2008; 2009).

O estresse hídrico normalmente diminui a porcentagem e a velocidade de germinação, mas existe grande variação entre as espécies, desde aquelas muito sensíveis até as mais resistentes. Os eventos subsequentes à germinação, como o estabelecimento e desenvolvimento de plântulas são também paralisados devido à limitação hídrica. Como estratégia, as células seminais geralmente promovem um acúmulo de compostos osmoticamente ativos, os quais atraem água para dentro da célula por diferença de potencial osmótico (BEWLEY & BLACK, 1994). A restrição hídrica apesar de reconhecidamente prejudicial ao processo de germinação, pode ser utilizado como ferramenta para aumento de vigor de sementes especialmente sob condições adversas. Tal técnica é conhecida como pré-condicionamento ou *priming*.

Priming

Tratamentos de *priming* envolvem controle da absorção de água pelas sementes, promovendo a iniciação de algumas atividades metabólicas inerentes ao processo germinativo, mas prevenindo a protrusão radicular (que finaliza o processo de embebição). Em seguida as sementes são secas, até atingirem seu conteúdo de água inicial. Os principais resultados observados após aplicação do *priming* são melhoria da emergência em campo, aumento da velocidade e uniformidade de germinação, quebra de dormência, além do aumento da tolerância a estresses (abióticos e bióticos) (VARIER et al., 2010; CHEN et al., 2012). Heydecker et al. (1973) foram os primeiros a descrever os efeitos positivos do priming em sementes. Alguns trabalhos também apontam para os efeitos negativos do *priming* em termos de comprometimento da longevidade (BRUGGINK et al., 1999; POWELL et al., 2000; VARIER et al., 2010).

O controle da embebição é feito através de diferentes técnicas, a saber: *osmopriming*, também conhecida como osmocondicionamento, a qual utiliza-se de algum agente osmótico (geralmente o polietilenoglicol por não ser tóxico a semente) para controlar a hidratação seminal; *halopriming*, que faz uso de alguma solução salina para controle da embebição e *hydropriming*, a qual utiliza-se da água para iniciar alguns eventos germinativos, mas prevenindo a germinação *strictu sensu* (GHASSEMI-GOLEZANI et al., 2008). Durante o *priming* as sementes absorvem água na fase I e II promovendo processos de reparo no DNA e mitocôndrias, assim como síntese de novos RNAs e proteínas (Figura 2). A grande diferença do *priming* para o processo de germinação normal é que a taxa de absorção de água é muito mais lenta e controlada (VARIER et al., 2010).

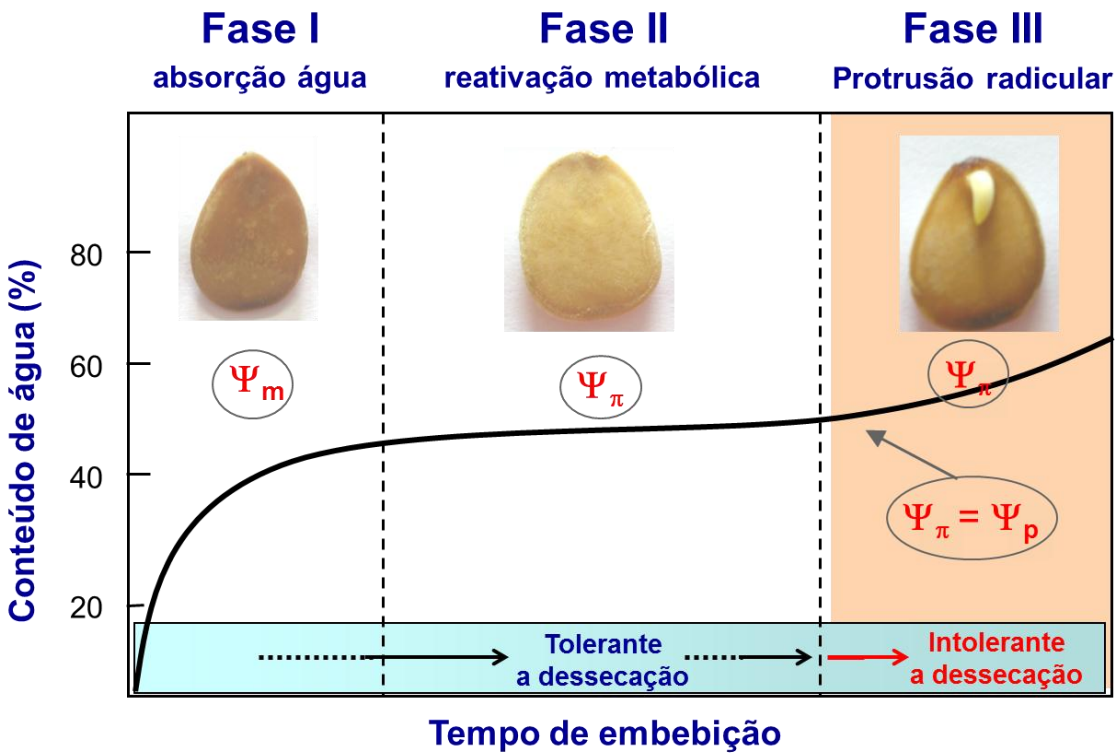

Figura 2. Padrão trifásico da germinação de sementes, ilustrado por três fases distintas (fase I = embebição rápida para ativação metabólica, fase II = embebição mínima, investimento em reparo e síntese de biomoléculas, fase III = retomada da embebição, crescimento embrionário). Adaptado de Bewley and Black, 1994.

Alguns trabalhos têm evidenciado alguns eventos específicos da condição de priming. Gallardo et al. (2001) por exemplo, demonstrou através de um estudo de proteômica em *Arabidopsis thaliana*, algumas proteínas estritamente expressas em condição de *priming* (como β-tubulina, subunidade β da proteína de armazenamento 12S-2 e Heat Shock Protein - HSP). Yacoubi et al. (2011) identificou proteínas com expressão diferencial (como HSPs e Glutationa S-transferase) em condições de *priming* em *Medicago sativa*. Weibrecht et al. (2011) propuseram que ativação da respiração e rápida produção de ATP parecem ser eventos metabólicos primários induzidos pelo *priming*. Powell et al. (2000) observou um aumento na proporção de DNA nuclear 4C e no acúmulo de β-tubulina em sementes de couve-flor de alto vigor submetidas a tratamento de hidratação aerada.

Medidas de vigor em sementes osmocondicionadas

Partindo-se do pressuposto que o osmocondicionamento aumenta a tolerância a estresses, assim como pode comprometer a longevidade de sementes, vários parâmetros fisiológicos podem ser utilizados para validar tais princípios, dentre eles: teste do envelhecimento acelerado, análise de crescimento de mudas oriundas de sementes osmocondicionadas, avaliação da tolerância cruzada e avaliação da longevidade de sementes (através do armazenamento).

O teste do envelhecimento acelerado foi desenvolvido a fim de se determinar o potencial de armazenamento de sementes, baseando-se no pressuposto de que a taxa de deterioração das mesmas é aumentada pela exposição a condições de elevada temperatura e umidade relativa (GARCIA et al., 2004; BRASIL, 2009; PINHO et al., 2010; CORTE et al., 2010). Nestas condições, o uso do osmocondicionamento pode ser útil para aumento do vigor e da tolerância a estresses em sementes, contendo assim, o processo de deterioração.

A análise de crescimento de mudas obtidas a partir de sementes osmocondicionadas é outro parâmetro funcional para avaliar o efeito do osmocondicionamento na emergência em campo e no desenvolvimento de plântulas. Segundo Marcos-Filho (2005) tal técnica pode elevar o índice de emergência das mudas, além de sincronizar a germinação de sementes. Além disso, pode proporcionar maior desenvolvimento das plântulas, estimulando o crescimento da parte aérea e dando maior rapidez no amadurecimento. Trabalhos como o de Ghassemi-Golezani et al. (2008) com lentilha, Maiti et al. (2009) com algumas hortaliças e de Suñé et al. (2002) com *Adesmia latifolia* comprovam os efeitos benéficos do pré-condicionamento na emergência e no desenvolvimento de plântulas em campo.

A exposição de sementes a determinados agentes osmóticos que promovem o osmocondicionamento (como o polietilenoglicol), ou mesmo o processo de secagem posterior ao processo, podem ser considerados fatores de estresse iniciais que podem ativar uma série de genes, criando uma espécie de "memória" vegetal àquele dado estresse. Numa possível nova exposição, a resposta a este mesmo estresse será mais rápida e potencializada (BRUCE et al., 2007; CHEN et al., 2012). Outra vertente associada ao processo é o que chamados de tolerância cruzada, onde a exposição a um determinado estresse específico (por exemplo, o *priming*) pode desencadear respostas a outros tipos de estresses secundários (RIZHSKY et al., 2002).

O estudo da longevidade em sementes osmocondicionadas também é objeto de interesse de muitos pesquisadores, visto que a literatura reporta o osmocondicionamento e seu consequente processo de secagem como agentes comprometedores da viabilidade de sementes armazenadas, devido ao avanço do processo germinativo que pode culminar na perda da tolerância a dessecação (POWELL et al., 2000; BUTLER et al., 2009). Uma vez que as sementes estejam próximas ao estádio III da germinação, a taxa de deterioração será mais alta em sementes pré-condicionadas e armazenadas (VARIER et al., 2010).

Ferramentas moleculares utilizadas para estudo do aumento vigor propiciado pelo priming

Dentre as ferramentas utilizadas para estudo dos genes responsivos ao osmocondicionamento, a Hibridização Supressiva Subtrativa (SSH) assume um papel importante, visto que é um método amplamente utilizado para separação de moléculas de DNA que distingue duas amostras de DNA distintas. As duas principais aplicações da SSH são subtração do cDNA e subtração do DNA genômico. A técnica de SSH é baseada primariamente na supressão por PCR, combinada com um procedimento simples de normalização e subtração. Os passos de normalização equalizam a abundância dos fragmentos de DNA dentro da população alvo, e os passos da subtração excluem sequências que são comuns as populações que estão sendo comparadas. Tal procedimento aumenta a probabilidade de obtenção de cDNAs ou fragmentos de DNA de baixa abundância (que são diferencialmente expressos) e simplifica a análise das bibliotecas subtraídas (DIATCHENKO et al., 1996; LUKYANOV et al., 2007). As várias etapas da SSH estão esquematizadas na figura 2.

A hibridização supressiva subtrativa tem sido utilizada para identificação de genes diferencialmente expressos em espécies vegetais cultivadas submetidas a condições de estresses abióticos, como por exemplo, déficit hídrico ou osmocondicionamento. Clement et al. (2008) identificaram genes diferencialmente expressos em nódulos de *Glycine max*, submetidos a condições de deficiência hídrica, utilizando-se a técnica de SSH; Jiang et al. (2004) encontraram genes com expressão diferencial relacionados ao estresse hídrico em plântulas de *Haloxylon ammodendron*; Porth et al. (2005) através da técnica de SSH, observaram que vários genes foram induzidos pelo estresse osmótico aplicado em uma cultura de células oriunda de *Quercus petraea* e Cortez-Baheza et al. (2007) identificaram genes diferencialmente expressos em sementes *Capsicum annuum* osmocondicionadas.

Estudos voltados a identificação de genes responsivos a restrição hídrica em sementes de espécies nativas (como *Poincianella pyramidalis*) ainda são escassos.

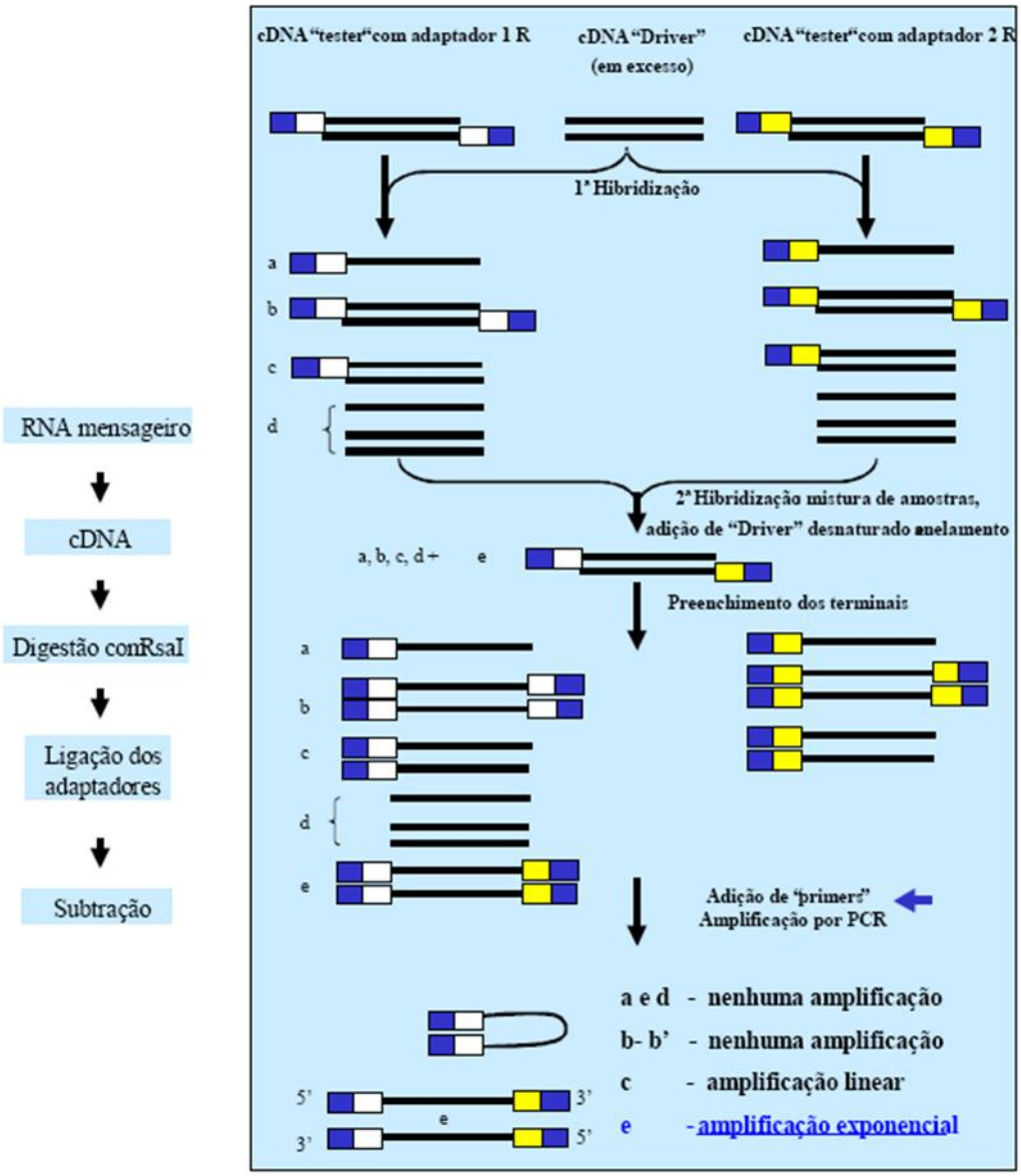

Figura 2. Diagrama esquemático do processo de subtração do cDNA. Linhas sólidas representam cDNA tester (alvo) e driver (controle) digeridos pela enzima de restrição Rsa I. Caixas sólidas representam a região externa dos adaptadores 1 e 2R que correspondem a sequência do PCR *primer* 1 utilizado na PCR. Caixas claras representam a parte interna do adaptador 1 a sequência correspondente do Nested PCR *primer* 1. Caixas sombreadas representam a parte interna do adaptador 2R e a sequência correspondente do Nested PCR *primer* 2R. Fonte: ABREU, 2007.

A técnica de SSH pode apresentar algumas limitações quanto ao processo de subtração devido a alguns fatores, dentre eles: números de genes diferentes entre as duas condições em comparação, abundância relativa dos transcritos, nível de indução do gene em questão, magnitude dos níveis basais de expressão dos genes, além do enriquecimento e normalização das bibliotecas (REBRIKOV & KOGAN, 2003). Dessa forma faz-se necessário o uso de uma técnica complementar, para validação dos resultados obtidos com a SSH.

A técnica de qRT-PCR (*Quantitative reverse transcription - polymerase chain reaction*) é uma alternativa interessante de validação, uma vez que mede o nível de expressão do gene de interesse em tempo real. Além disso, devido a alta sensibilidade, permite a quantificação de transcritos raros e detecção de pequenas variações na expressão gênica. Tal detecção é feita por monitoramento do aumento da intensidade de fluorescência de um fluoróforo específico (geralmente SYBR Green I), a qual se correlaciona com o aumento da concentração do produto de PCR. Geralmente dois métodos de quantificação são utilizados: o método de quantificação relativa e o método de quantificação absoluta. Para análises de mudanças fisiológicas nos níveis de expressão gênica (como aquelas induzidas pelo osmocondicionamento) a quantificação relativa é a mais recomendada, visto que compara a expressão relativa de um gene alvo contra um gene referência. Os ensaios de quantificação relativa assumem que os genes referência são expressos de maneira constitutiva em um amplo espectro de condições, e a estabilidade de expressão dos mesmos deve ser provada, antes dos mesmos serem escolhidos para normalização dos dados de qRT-PCR. O uso de vários genes referência tem sido cada vez mais requisitado para normalização destes dados (PFAFFL et al., 2001; REID et al., 2006; REGIER et al., 2010).

CHAPTER 1

PHYSIOLOGICAL CHARACTERIZATION OF *Poincianella pyramidalis* (TUL.) L. P. QUEIROZ SEEDS UNDER ABIOTIC STRESSES CONDITIONS

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Abstract: (**Physiological characterization of** *Poincianella pyramidalis* **(Tul.) L. P. Queiroz seeds under abiotic stresses conditions**). Environmental stresses as drought, salinity, cold and heat cause adverse effects on plant growth and yield through modifications at morphological, biochemical and molecular levels. Species from arid and semi-arid areas are exposed to extreme environmental conditions and the study of their behavior, especially during the first stages of life where abiotic stresses directly influence seed and seedling quality, should not be neglected. In this study we evaluated seed germination and seedling establishment of *Poincianella pyramidalis* (catingueira) under different osmotic potentials and temperatures. Catingueira seeds were submitted to a range of constant temperatures (20, 25, 30, 35, 37 and 40 ºC) to check the optimal temperature for seed germination. Additionally seeds and seedlings were soaked in different osmotic conditions (0.0; -0.2; -0.4; -0.6; -0.8; -1.0 and -1.2 MPa). According to our results, *P. pyramidalis* can germinate well under a wide range of temperatures and osmotic potentials and also keep up development under adverse conditions due its ecological plasticity and moderate tolerance to osmotic stress.
Key-words: Seed quality; PEG 8000; Temperature; Catingueira.

Resumo: (**Caracterização fisiológica de sementes de** *Poincianella pyramidalis* **(Tul.) L. P. Queiroz sob condições de estresses abióticos**). Estresses ambientais como seca, salinidade, frio e calor causam efeitos adversos no crescimento e rendimento de plantas através de modificações aos níveis morfológico, bioquímico e molecular. Espécies de regiões áridas e semi-áridas são expostas a condições ambientais extremas e o estudo do comportamento delas, especialmente nos primeiros estádios de vida onde estresses abióticos afetam diretamente a qualidade das sementes e plântulas, não devem ser negligenciados. Neste estudo nós avaliamos a germinação de sementes e o estabelecimento de plântulas de *Poincianella pyramidalis* (catingueira) sob diferentes potenciais osmóticos e temperaturas. Sementes de catingueira foram submetidas a uma faixa de temperaturas constantes (20, 25, 30, 35, 37 and 40ºC) para checar a temperatura ótima para germinação das sementes. Além disso, sementes e plântulas foram mantidas em diferentes condições osmóticas (0,0; -0,2; -0,4; -0,6; -0,8; -1,0 and -1,2 MPa). De acordo com nossos resultados, *P. pyramidalis* pode germinar bem em uma ampla faixa de temperaturas e potenciais osmóticos e manter seu desenvolvimento sob condições adversas devido a sua plasticidade ecológica e sua tolerância moderada ao estresse osmótico.
Palavras-chave: Qualidade de sementes; PEG 8000; Temperatura; Catingueira.

INTRODUCTION

Among the abiotic stresses that cause adverse effects in plant growth and development in arid and semi-arid regions, drought and heat seem to be the most important (BRAY et al., 2000; WANG et al., 2001).

Under water deficit condition, protective mechanisms against desiccation are developed (BEWLEY & OLIVER, 1992), but a severe water stress results in metabolic imbalance (BLACKMAN et al., 1992). In this context Larcher (2000) proposed that knowledge about how the stress affects the germination process has special

ecophysiological importance for the evaluation of stress tolerance and understanding of the adaptation level of plant species.

To simulate osmotic stress (in lab conditions), most commonly germination studies have been carried out by adding solutions prepared from inorganic (NaCl, KNO_3 and $MgSO_4$) and organic (mannitol, sucrose and polyethyleneglycol (PEG)) osmotic agents (KISSMAN et al., 2010). Among them PEG (6000, 8000 and 20000) has been widely used, because it has no additional adverse effects on the seeds, since it is chemically inert, nontoxic and cannot enter the seed due to its high molecular weight. PEG can impose different water restriction to the seed, depending on its concentration (BEWLEY & BLACK, 1994; BRACCINI et al., 1996; VILLELA et al., 2001; KISSMAN et al., 2010).

Temperature affects a broad spectrum of cellular components and metabolism, and extreme temperatures impose stresses of variable severity which depends on the rate of temperature change, intensity and duration (SUNG et al., 2003). Considerable effort has been devoted to understand the molecular basis of resistance to heat and it has been revealed that plants react to temperature variations by alterations in metabolic rates, protein turnover, production of osmolytes, membrane function and gene expression (STUPNIKOVA et al., 2006).

The abiotic stresses mentioned will have a direct influence on seed quality, which is a complex trait that comprises many different attributes describing the condition of a seed lot. These attributes include germination characteristics, dormancy, seed and seedling vigour, uniformity in seed size, storability, absence of mechanical damage, as well as the ability to develop into a normal plant (DICKSON, 1980; HILHORST & TOOROP, 1997; LIGTERINK et al., 2012). Seed quality is established during seed development and maturation, as a result of interactions between the genome and the environment. This mechanism is part of the normal adaptation of plants to a varying environment and it is aimed at maximizing the probability of successful offspring (HUANG et al., 2010; LIGTERINK et al., 2012).

In this study we evaluated the seed quality of *Poincianella pyramidalis* in different thermic and osmotic conditions. Commonly known as catingueira, the mentioned species is a legume from Brazilian Northeast semi-arid, largely cited on systematic survey approaches in Caatinga areas. *P. pyramidalis* shows strong adaptability to Caatinga adversities (drought and heat for example), what may be confirmed by its high level of endemism. Due its multiple potential it is highly used by the local population: its wood has commercial value, its bark has anti-diarrheal properties and their leaves are used as forage

to the cattle during drought period (SAMPAIO & RODAL, 2000; MAIA, 2004; SALVAT et al., 2004).

MATERIALS AND METHODS

***Seed collection*:** The seeds were harvested from catingueira trees, located at Petrolina – PE, Brazil, under the following coordinates: 9º 30' 21" S 40º 30' 21" W in September 2008. Petrolina has an annual average air temperature above 24°C (TEIXEIRA, 2009). The climate of the region is the type Bswh', according to Köeppen classification, corresponding to a region of arid climate (RUBEL & KOTTEK, 2010). The thermic homogeneity strongly contrasts with the spatial and temporal heterogeneity of rainfall (TEIXEIRA, 2010). After harvest, the seeds were dried in the shade at room temperature, during three days, aiming to reduce their moisture content and thereby slowing deterioration and chemical modifications on seed tissues. Seeds were stored on the fridge (8 ± 2 ºC) in plastic bags, until start physiological assays.

***Thermic screening*:** Seeds of *P. pyramidalis* were treated with 0.5% NaClO solution for 5 minutes and placed to germinate in sterilized plastic boxes (11 x 11 x 3.5 cm) containing two layers of filter paper and 13 ml of distilled water. Germination test was performed at 20, 25, 30, 35, 37 and 40 °C under 12 hours photoperiod for nine days. Germinated seeds were determined by counting the number of individual seeds that had a protruded radicle (2 mm) at each interval of 24 hours. The following parameters were evaluated: G_{max} (final maximal germination); t_{50} (time to 50% germination, germination rate) and U_{8020} (time between 20 and 80% of germination, germination uniformity) (ISTA, 2011; (Germinator software - JOOSEN, 2010)).

***Osmotic screening and seedling growth*:** To simulate water restriction, polyethyleneglycol 8000 solutions were prepared in different osmotic potentials (-0.2; -0.4; -0.8; -1.0; -1.2 MPa), according the table cited by Villela et al. (2001) based on a formula from Michel (1983) and Michel et al. (1983). Water (0.0 MPa) was considered as control. Germination test was performed at 25 °C (optimal temperature previously determined by the thermic assays) under 12 hours photoperiod for nine days. Germinated seeds were determined by counting the number of individual seeds that had a protruded radicle (2 mm) at each interval of 24 hours. The following parameters were evaluated: G_{max}; t_{50} and U_{8020}

(Germinator software - JOOSEN, 2010). Seedling development was evaluated by percentage of normal seedlings (seedlings emerged after 8 days of imbibition with launched leaves, developed root and shoot, cap of root with no oxidation and absence of deformations in cotyledons), percentage of abnormal seedlings (seedlings emerged after 8 days of imbibition with troubles in any one of those parameters established for normal seedlings), percentage of inviable seeds (deteriorated seeds), percentage of not germinated seeds and normal seedling vigour (number of leaves, length of roots and shoots, dry matter of roots and shoots and shoot/root ratio). To get dry matter measurements, seedlings were packed in paper bags and kept in an oven with forced ventilation, set at 60 °C till constant weight (BRASIL, 2009; ISTA, 2011).

***Experimental design and Statistical analysis*:** The experimental design was completely randomized with four replicates of 25 seeds and a statistical analysis was made by comparing means using Tukey's test at 5% probability through the statistical package SISVAR 4.3 (FERREIRA, 1999). The germination and seedling data expressed in percentage were transformed using the respectively functions: $(x+0,5)^{0,5}$ and arc sine$\sqrt{x}/100$.

RESULTS AND DISCUSSION

Determining the optimal germination temperature for catingueira seeds

Temperature heavily influenced the germinative performance of catingueira seeds. Germination was observed in all tested temperatures, showing a big ecological plasticity of catingueira. At 20ºC, final germination percentage was 95%, at 30 ºC it was 93%, at 25 ºC it was 96% and at 35 ºC start to decrease. At 25 and 30 °C germination started after 12 hours of imbibition, at 20 ºC the seed germination started after 24 hours of imbibition and at the others temperatures the germination started later (Figure 1).

According to Borges & Rena (1993), the optimal temperature for germination are quite variable among forest species, and most tropical and subtropical species require temperatures ranging from 20 to 30 °C, depending on the normal temperature regimes in their local regions. This report confirms our findings, since we found higher germination percentage for catingueira seeds from 20 to 30 ºC. For some others legumes as *Dinizia excelsa* (VARELA et al., 2005) and *Caesalpinia ferrea* (LIMA et al., 2006) a similar pattern was observed under constant temperatures (20 to 30 ºC).

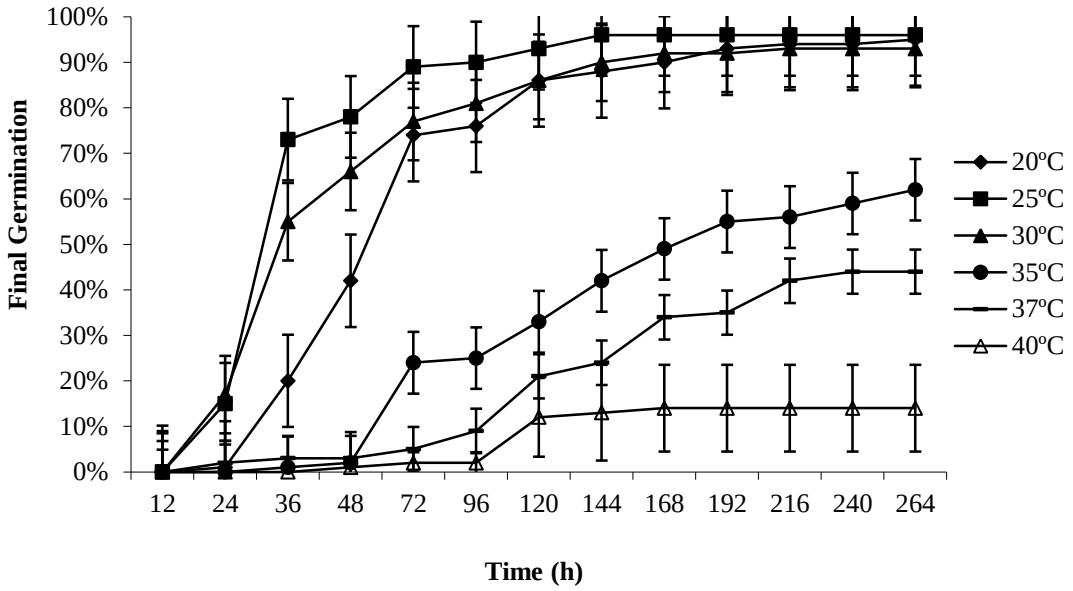

Figure 1. Cumulative germination curves of *Poincianella pyramidalis* seeds at different temperatures.

There were no significant differences in final germination percentage between germination at 20 ºC (95%), 25 ºC (96%) and 30 ºC (93%), but those values were statistical higher than the ones at 35 ºC (62%), 37 ºC (44%) and 40 ºC (14%) (Figure 2). *Parkia pendula* seed germination also did not significantly differ between 20, 25 and 30 °C, showing the great capacity of legume species to germinate at a wide range of temperatures (ROSSETO et al., 2009). The drop on seed germination at 40 ºC could be probably explained by the effect of high temperatures on the cells increasing fluidity of membrane lipids and decreasing the strength of hydrogen bonds leading to leakage of ions. They can also compromise the three-dimensional structure of enzymes (TAIZ & ZEIGER, 2010) what might be negatively influencing the germinative process.

Concerning the effect of temperature on t_{50} (germination rate), the same pattern as in the final germination percentage was observed: statistically faster germination at 20 ºC (50.8h), 25 ºC (30.9h) and 30 ºC (32.2h) compared to germination at 35 ºC (109.3h), 37 ºC (132.4h) and 40 ºC (108.0h). The uniformity (U_{8020}) was highest (= lowest U_{8020}) at 25 ºC (14.91h) and turned out to be the optimal temperature for catingueira seed germination (Figure 2).

The ideal temperature is one of the factors that promote the most efficient combination of percentage and speed germination in a short available period, influencing

the water uptake as well as the metabolic and biochemical reactions that regulate those processes (BEWLEY & BLACK, 1994; CARVALHO & NAKAGAWA, 2000; MARCOS FILHO, 2005). This ideal temperature could be associated with the ecological characteristics of each species (SOUSA-SILVA et al., 2001).

Ferraz-Grande et al. (2006) studying the germination of *Caesalpinia peltophoroides* seeds also observed the best rates (germination and speed germination) at 25 ºC. Araújo Neto et al. (2003) analyzing *Acacia polyphylla* seed germination also found 25 ºC as the best temperature on their assays. In *Parapiptadenia rigida* seeds, Mondo et al. (2008) concluded that the germination test must be performed at 25 ºC. Jose et al. (2011) studying *Apuleia leiocarpa* (Fabaceae) seed germination observed higher final germination percentage and speed germination at 30 ºC. The uniformity, however, was higher at 25 ºC as in catingueira seeds. Pacheco et al. (2010) analyzing the germination of *Dimorphandra mollis* seeds at different temperatures also found better results associated to high temperatures (30 and 35 ºC), but even though within the range expected for tropical and subtropical species.

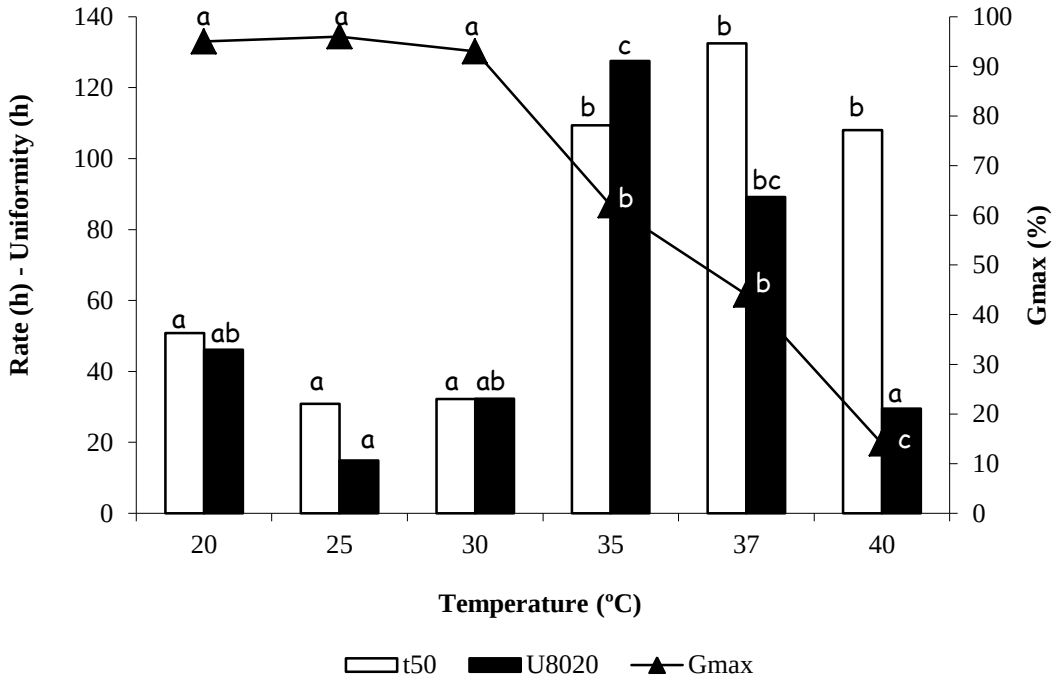

Figure 2. G_{max} (Final maximal germination), t_{50} (germination rate) and U_{8020} (germination uniformity) of *Poincianella pyramidalis* seeds under different temperatures. Averages with the same letters do not differ significantly by the Tukey test at 5% probability.

Effect of osmotic stress on germination of catingueira seeds

It was observed decrease of catingueira seed germination when there was a reduction of the osmotic potential of the PEG solutions. After 12 hours we observed germination of the first seeds in PEG solutions at 0.0 MPa and -0.2 MPa. Seeds imbibed in a PEG solution of -0.4 MPa started germination after 24 hours. The ones imbibed at -0.6, -0.8 and -1.0 MPa PEG solution started to germinate after 48, 96 and 144 hours respectively (Figure 3). At -1.2 MPa no seed germination was observed.

In general, seeds integrate environment signals to determine the best moment to start radicle protrusion and the subsequent seedling development. This is critical for the seeds, because the seedling survival is dependent on the water availability in the medium (BRADFORD, 1997). Similar to the findings of Heydecker & Coolbear (1977), we observe that an increase in environmental stress intensity (as water restriction) leads to an increase in germination time, subsequently affecting the percentage of seed germination. Rosa et al. (2005) also found a delay on seed germination upon reduction of osmotic potential of PEG solutions for *Ateleia glazioviana* seeds.

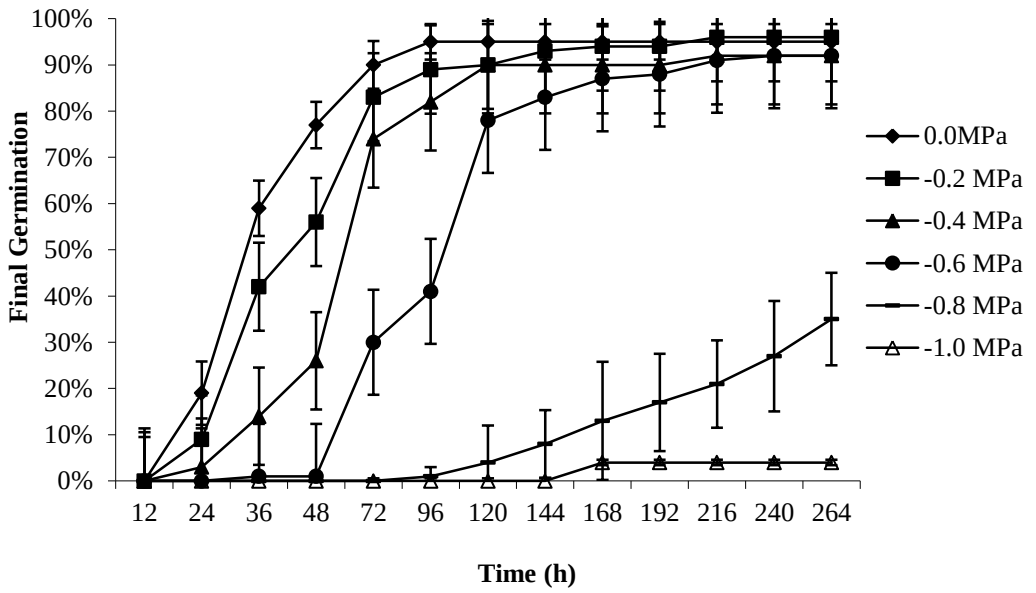

Figure 3. Cumulative germination curves of *Poincianella pyramidalis* seeds in different osmotic potentials of PEG solutions.

No significant differences in final germination percentage were detected between seeds incubated in PEG solutions at 0.0 MPa (95%); -0.2 MPa (96%); -0.4 MPa (92%) and

-0.6 MPa (92%), but those values were significant higher than those obtained for seeds incubated at -0.8 MPa (35%), -1.0 MPa (2%) and -1.2 MPa (0%) (Figure 4). The reduction in germination percentage associated with the increase of osmotic stress might be explained by the effect of PEG, which allows seeds to start imbibition, until equilibration with the osmotic potential of the external solution. At that point further water uptake is prevented as well as subsequent radicle protrusion (FONSECA et al., 2003).

Antunes et al. (2011) evaluated a seed lot of *Poincianella pyramidalis* from 2007 stored for 0, 3 and 6 months and observed high germination percentages at 0.0 to -0.6 MPa PEG. In *Bowdichia virgilioides*, seed germination percentage was high at 0.0 to -0.3 MPa PEG, and drastically decreased from -0.5 MPa PEG downwards (SILVA et al., 2001). *Cnidosculus juercifolius* seeds showed a same pattern for germination at 0.0, -0.3 and -0.5 MPa PEG (SILVA et al., 2005). Chauhan & Johnson (2009) analyzed the behavior of *Mimosa pudica* seeds under osmotic stress (0.0 to -1.0 MPa PEG) and found a decrease of seed germination from -0.4 MPa PEG downward, indicating a lower tolerance to water stress than catingueira seeds. *Mimosa invisa* seeds showed a drop of germination from -0.8 MPa PEG downwards with no germination at -1.2 MPa PEG (CHAUHAN & JOHNSON, 2008) as in catingueira seeds.

The t_{50} was higher for seeds that were subjected to higher osmotic potentials (0.0MPa/32.58 hours; -0.2MPa/41.32 hours; -0.4MPa/56.81 hours) and was lower to seeds imbibed in lower osmotic potentials (-0.6MPa/92.73 hours; -0.8MPa/233.54 hours; -1.0MPa/90.04 hours) (Figure 4). The time to attain 50% germination demonstrates the vigour of seeds, since it is related to kinetic criteria under suboptimal conditions (CORBINEAU, 2012). Others authors as Delachiave & Pinho (2003) studying *Senna occidentalis* seeds, observed the same delay of seed germination when there was reduction of the osmotic potential.

At -1.0 MPa PEG we observed the highest uniformity, what can be explained by the few vigorous seeds that quickly germinate on that condition. There were not significant differences in uniformity for seed germination from 0.0 to -0.4 MPa PEG. The uniformity of germination measures the variability among seeds in relation to the mean germination time of the sample (BEWLEY & BLACK, 1994; RANAL & SANTANA, 2006), thus when the osmotic stress increases, seeds require more time to germinate and there is a tendency to enlarge the germination along the time.

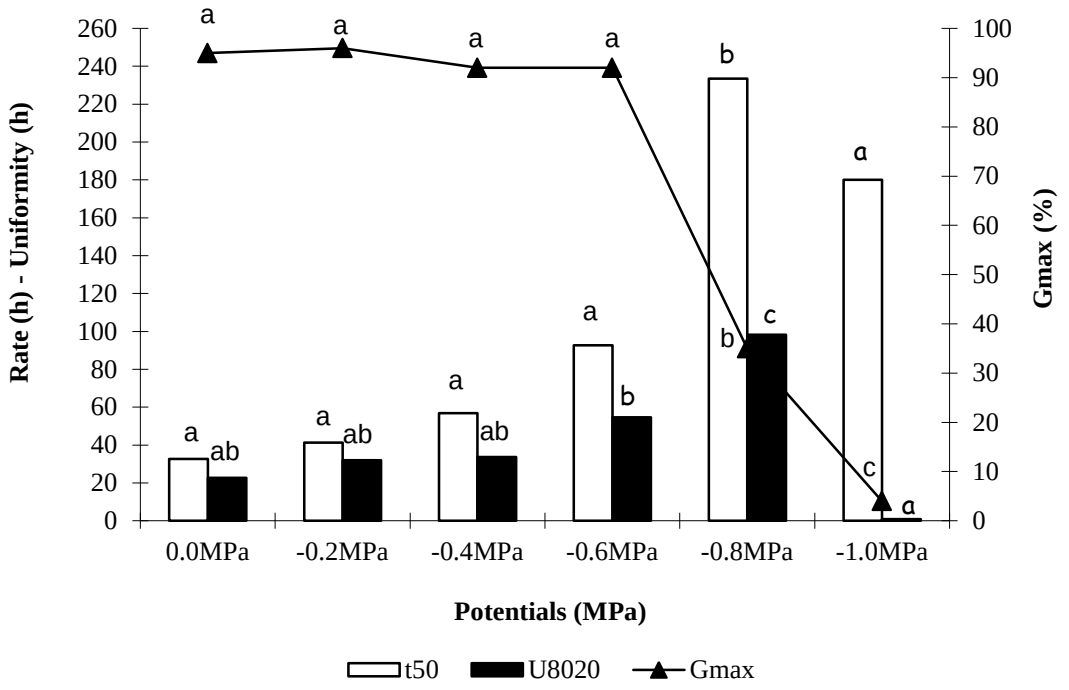

Figure 4. G_{max} (Final maximal germination), t_{50} (germination rate) and U_{8020} (germination uniformity) of *Poincianella pyramidalis* seeds in different osmotic potentials of PEG solutions. Averages with the same letter do not significantly differ by the Tukey test at 5% probability.

Effect of osmotic stress on catingueira seedling growth

Although germination tests are usually carried out under near-optimal conditions for germination, giving information about the viability of a seed population, they cannot predict seedling performance under sub-optimal conditions (CORBINEAU, 2012). The analysis of seedling growth provides additional data that complement germination tests, allowing that diverse degrees of vigour might be distinguished. More vigourous seeds would result in greater seedlings (showing higher length and dry matter for example) (AOSA, 1983; PIÑA-RODRIGUES et al., 2004; PACHECO et al., 2011).

As expected, a higher percentage of normal seedlings (with launched leaves, developed root and shoot, cap of root with no oxidation and absence of deformations in cotyledons) was observed in higher osmotic potentials (0.0 MPa and -0.2 MPa) of PEG solutions. From -0.4 MPa PEG downwards, there was a drastic decrease of normal seedlings showing that seedling growth is more sensitive to water stress than seed germination. From -1.0 MPa PEG downwards was not observed normal seedlings. In

parallel, an increase of abnormal seedlings (seedlings with troubles in any parameter mentioned for normal seedlings) was observed until -0.6 MPa (Table 1). From -0.8 MPa downwards there was a reduction in abnormal seedlings percentage and in inviable seeds percentage, probably explained by the effect of osmotic adjustment adopted by the seeds as a protection strategy. The percentage of non-germinated seeds increased with the increasement of osmotic stress, achieving 98 and 100% at -1.0 and -1.2 MPa. Those seeds were moved to water to check germination and it was found 98% and 100% of germination respectively (data not showed).

Table 1. Normal seedlings percentage (% NS), Abnormal seedlings percentage (% AS), Inviable seeds percentage (% IS) and Not germinated seeds percentage (% NGS) of *Poincianella pyramidalis* seeds under different osmotic potentials.

Potentials	**Parameters ***			
(MPa)	**% NS**	**% AS**	**% IS**	**% NGS**
0.0	60a	35c	3a	2c
-0.2	68a	28c	4a	1c
-0.4	27b	64b	4a	5c
-0.6	2c	88a	5a	5c
-0.8	3c	32c	0a	65b
-1.0	0c	2d	0a	98a
-1.2	0c	0d	0a	100a

***** Averages with the same letter do not differ by the Tukey test at 5% probability.

Reduction of osmotic potential of PEG solutions resulted in a decrease in all seedling vigour parameters analyzed. No significant differences could be detected for number of leaves, root length and shoot length of catingueira seedlings at 0.0, -0.2 and -0.4 MPa PEG (Table 2).

Root and shoot weight of catingueira seedlings were significant higher at 0.0 and -0.2 MPa PEG, showing an investment in biomass when water is freely available (low PEG concentrations). The ratio shoot/root weight demonstrated more allocation of biomass on the shoot part for seedlings kept under osmotic solutions with high water availability (0.0; -0.2 and -0.4 MPa PEG) and little more investment in biomass on the root part for seedlings cultivated on osmotic solutions with high PEG concentration (-0.6 and -0.8 MPa) (Table 2).

Similar as our data, Safarnejad (2008) studying vigour parameters in *Medicago sativa* seedlings (from different accessions) kept under different osmotic potentials,

verified a reduction on number of leaves, root and shoot lengths, root and shoot dry matter and ratio shoot/root under increasing PEG concentrations. Also Perez & Nassif (1998) observed a decrease in seedling size, dry weight and ratio shoot/root weight under increasing PEG concentrations for algarobeira (*Prosopis juliflora*) seedlings.

Table 2. Number of leaves (NL), Root length (RL), Shoot length (SL), Root weight (RW), Shoot weight (SW) and Ratio shoot/root weight (S/R) of normal *Poincianella pyramidalis* seedlings after 8 days of imbibition under different osmotic potentials.

Potentials	**Parameters ***					
(MPa)	**NL**	**RL (cm)**	**SL (cm)**	**RW (g)**	**SW (g)**	**S/R**
0.0	1.89a	4.33a	7.26a	0.143a	1.338a	9.41ab
-0.2	1.80a	4.49a	7.02a	0.165a	1.604a	9.90ab
-0.4	1.68a	4.36a	5.71a	0.060b	0.609b	11.00a
-0.6	0.5b	1.27b	1.71b	0.007bc	0.043c	5.04abc
-0.8	0.5b	1.27b	1.55b	0.005c	0.039c	2.07bc
-1.0	0.0b	0.0b	0.0b	0.0c	0.0c	0.00c
-1.2	0.0b	0.0b	0.0b	0.0c	0.0c	0.00c

***** Averages with the same letter do not differ by the Tukey test at 5% probability.

CONCLUSIONS

We conclude that *P. pyramidalis* seeds germinate on a wide range of temperatures (20 ºC to 40 ºC), demonstrating great ecological plasticity. They tolerate moderate level of osmotic stress, since they germinated until -1.0 MPa PEG and the seedlings are lesser tolerant to osmotic stress with almost no normal seedlings at -0.6 MPa PEG or lower.

From the data acquired with the temperature and PEG curves it is possible to define further the best conditions to perform priming of catingueira seeds (-1.2 MPa at 25 ºC) and also to investigate those effects on the seeds at molecular level.

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CHAPTER 2

OSMOPRIMING OF *Poincianella pyramidalis* (TUL.) L. P. QUEIROZ SEEDS

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Abstract: (**Osmopriming of** *Poincianella pyramidalis* **(Tul.) L. P. Queiroz seeds**). Osmopriming is a seed enhancement method that involves control of water uptake been largely used to improve germination, emergence and seedling growth. Based on these principles we proposed in this approach investigate the effect of osmopriming on *P. pyramidalis* (catingueira) seed germination, longevity, tolerance to stresses and establishment. Firstly catingueira seeds were osmoprimed in polyethyleneglycol 8000 (-1.2 MPa) for seven days at 25 ºC. After that the following assays were performed: germination test at 25 ºC; accelerated ageing (at 40 and 45 ºC) and natural ageing to check seed longevity; induction of osmotic and thermic stresses to evaluate tolerance to stress after osmopriming and growth analysis of catingueira seedlings (obtained from unprimed and osmoprimed seeds) grown in greenhouse. Osmopriming improved final germination, germination rate and germination uniformity of catingueira seeds. Accelerated ageing at 40 ºC was reverted by osmopriming but at 45 ºC the combination between high temperature, high relative humidity and osmopriming promoted a negative effect on seed germination. Osmoprimed seeds packed in plastic bags and stored for two years on the fridge kept high germination percentage as in newly collected seeds. Stressful conditions as high temperature and severe osmotic stress compromised germination process and osmopriming could not recover it. There were no differences in growth among catingueira seedlings obtained from osmoprimed or unprimed seeds. We suggest that osmopriming is interesting to improve seed quality but an older catingueira seed lot probably will present a better response to the osmopriming.
Key-words: Catingueira, tolerance, growth, longevity, germination.

Resumo: (**Osmocondicionamento de sementes de** *Poincianella pyramidalis* (**Tul.**) **L. P. Queiroz**). Osmocondicionamento é um método de aumento de vigor de sementes que envolve o controle da absorção de água, sendo largamente utilizado para aumentar germinação, emergência e crescimento de plântulas. Baseado nestes princípios nós propusemos neste estudo investigar o efeito do osmocondicionamento na germinação de sementes, longevidade, tolerância a estresses e estabelecimento de *P. pyramidalis* (catingueira). Primeiramente sementes de catingueira foram osmocondicionadas em polietilenoglicol 8000 (-1,2 MPa) por sete dias a 25ºC. Posteriormente os seguintes ensaios foram realizados: teste de germinação a 25ºC; envelhecimento acelerado (a 40 e 45ºC) e envelhecimento natural para checar a longevidade das sementes; indução de estresse osmótico e estresse térmico para avaliar a tolerância das sementes a estresse depois do osmocondicionamento e análise de crescimento das plântulas de catingueira (obtidas a partir de sementes não osmocondicionadas e osmocondicionadas) crescidas em casa de vegetação. O osmocondicionamento aumentou a germinação final, a velocidade de germinação e a uniformidade da germinação de sementes de catingueira. O envelhecimento acelerado a 40ºC foi revertido pelo osmocondicionamento, mas a 45ºC a combinação entre alta temperatura, elevada umidade relativa e osmocondicionamento promoveu um efeito negativo na germinação das sementes. Sementes osmocondicionadas acondicionadas em sacos plásticos e armazenadas em geladeira por dois anos mantiveram alta porcentagem de germinação assim como as sementes recém-coletadas. Condições estressantes como alta temperatura e estresse osmótico severo comprometeram o processo germinativo e o osmocondicionamento não foi capaz de recuperar tal processo. Não houve diferenças no crescimento de plântulas de catingueira obtidas de sementes osmocondicionadas ou não osmocondicionadas. Nós sugerimos que o osmocondicionamento é interessante para aumentar a qualidade das sementes, no entanto, um lote mais velho de sementes provavelmente irá apresentar uma melhor resposta ao osmocondicionamento.
Palavras-chave: Catingueira, tolerância, crescimento, longevidade, germinação.

INTRODUCTION

Priming is a technique which involves uptake of water by the seed followed by drying. The earlier events of germination initiate but emergence of the radicle is prevented to avoid the loss of desiccation tolerance that is needed for subsequent drying, storage and marketing of them. Priming benefits depends on various factors among them: seed deterioration stage, duration and temperature of treatment, seed size, water absorption speed (associated with external matric and osmotic potentials), seed hydration level, drying post treatment and number of cycles drying/hydration (MATTHEWS et al., 1988; BASU et al., 1994; McDONALD, 2000; LARS, 2000; SOEDA et al., 2005; KISSMANN et al., 2010; VARIER et al., 2010).

Priming allows seeds rapid, uniform and increased germination, improved seedling vigour and growth under a broad range of environments resulting in better stand establishment (MATTHEWS et al., 1988; BASU et al., 1994; McDONALD, 2000; VARIER et al., 2010). To prevent radicle protrusion, water uptake may be limited by imbibition in an osmotic solution such as polyethyleneglycol (osmopriming), salt solution (halopriming) and water (hydropriming). Osmopriming contributes to significant improvement in seed germination in different plant species (POWELL et al., 2000; SOEDA et al., 2005; GHASSEMI-GOLEZANI et al., 2008; CHEN et al., 2010; ZHANG et al., 2012), especially in forest species (BORGES et al., 1994; SUNE et al., 2002; KISSMANN et al., 2010).

A negative effect of priming is that longevity of primed seeds can be considerably lesser compared to that of unprimed seeds during dry storage (BRUGGINK et al., 1999; SOEDA et al., 2005). The reduction in longevity is associated with the desired increase in speed of germination (BRUGGINK et al., 1999). However, in some crop species seed longevity is improved by priming (POWELL et al., 2000; YEH & SUNG, 2008). It has been suggested that improvements in seed longevity after priming will be most apparent in deteriorated seed lots as in these seeds repair will be most effective (BUTLER et al., 2009).

An easy and fast way to verify seed longevity during priming is to perform accelerated ageing of seeds by exposure them to high temperature and high humidity for several days (McDONALD, 1999; MODARRESI & VAN DAMME, 2003; YEH & SUNG, 2008). Rapidly deteriorating seeds subjected to accelerated ageing show decline in ability to emerge into vigorous seedlings (MODARRESI & VAN DAMME, 2003; SILVA et al., 2006; BASAK et al., 2006). Some of the deleterious effects of accelerated ageing are

associated with increased lipid peroxidation and decreased anti-oxidative activity (SATTLER et al., 2004; BAILLY, 2004).

Even priming been a technique that provides improvement on germination and establishment, it is *per si* an osmotic stress to the seed. Thus if the seed it is exposed to priming (or other kind of stress), a cascade of molecular events is activated with the perception of stress and ends with the expression of a set of target genes by making use of routes and common components in response to stress. This phenomenon is known as cross tolerance and allows plants acclimate to several different stresses after exposure to a specific stress (PASTORI & FOYER, 2002; RIZHSKY et al., 2002; FOOLAD et al., 2003). In a review made by Bruce et al. (2007), priming was considered as a stress-exposure that could leave seeds with a 'stress-memory'. Thus, the improved stress tolerance during post-priming germination may be a manifestation of 'cross-tolerance' induced by priming (CHEN et al., 2012).

In the literature there are many examples of successful seed priming with crop species, but there is little information for forest species. An example of that is the Brazilian forest species *Poincianella pyramidalis* (known as catingueira), a leguminous from Caatinga largely used by local population due to its medicinal and forage properties (MAIA, 2004). We proposed in this approach investigate the effect of osmopriming on catingueira seed germination, establishment, longevity and tolerance to osmotic stress.

MATERIALS AND METHODS

***Seed collection*:** The seeds were harvested from catingueira trees, located at Petrolina – PE, Brazil, under the following coordinates: 9º 30' 21" S 40º 30' 21" W in September 2008. After harvest the seeds were dried in the shade at room temperature, during three days, aiming to reduce their moisture content and thereby slowing deterioration and chemical modifications on seed tissues.

***Seed moisture content*:** Moisture content of catingueira seeds was determined by drying them for 17 hours in an oven at 103 ºC (ISTA, 2011) and expressed as a percentage of the seed dry weight.

***Osmopriming*:** Catingueira seeds with 5.38% of moisture content were primed in a solution of polyethyleneglycol 8000 (PEG 8000) at -1.2 MPa for seven days, at 25 ºC, with

constant light, making use of a roller system. The osmotic potential was chosen based on the previous osmotic stress assays of catingueira seeds. On completion of treatment the seeds were removed and rinsed out in distilled water then dried for four days in an incubator at 20 ºC and 30% RH when they reach their initial moisture content (HEYDECKER & GIBBINS, 1978; POWELL et al., 2000; NASCIMENTO et al., 2001). The following parameters were evaluated: G_{max} (final maximal germination); t_{50} (time to 50% germination, germination rate), U_{8416} (time between 16 and 84% of germination, germination uniformity) (Germinator software - JOOSEN, 2010) (BRASIL, 2009; ISTA, 2011).

***Accelerated ageing*:** Accelerated ageing of osmoprimed and unprimed catingueira seeds was conducted in plastic boxes (11 x 11 x 3.5 cm) with a suspended wire mesh screen containing 40mL of distilled water. They were incubated at 40 and 45 ºC (96% RH) for 48 and 96 hours at the dark. Before and after ageing, moisture content of the seeds was analyzed. Seeds unaged were used as control to the test (ISTA, 2011). After accelerated ageing, seeds were evaluated as to final germination (G%), mean time of germination (MTG - hours) and germination speed index (GSI) (BRASIL, 2009).

***Natural ageing*:** Osmoprimed catingueira seeds were packed in plastic bags (semipermeable) and stored on the fridge at 8 ± 2 ºC for two years at Lab of Seed Germination, State University of Feira de Santana, Brazil. Unprimed seeds were also stored and used as control. After storage, seed longevity was evaluated by G%, MTG and GSI (BRASIL, 2009).

***Cross Tolerance*:** Firstly catingueira seeds were osmoprimed in PEG 8000 at -1.2 MPa for seven days at 25 ºC to induce a starting osmotic stress on the seeds. Thus, a subsample was incubated in thermic conditions considered as stressful (37 ºC and 40 ºC) and another subsample was put in osmotic stress conditions again (-0.8 and -1.2 MPa). The seeds remained in those conditions for ten days. Osmoprimed and unprimed seeds incubated at 25 ºC in water were the control treatment. The following parameters were evaluated: G% and GSI (BRASIL, 2009).

***Seedling analysis in greenhouse*:** Osmoprimed and unprimed catingueira seeds were sowed in pots containing composting + washed sand + soil (1:1:1) substrate (pH corrected

by P_2O_5) been kept in greenhouse at 60% of luminosity. Four seeds were sowed per pot and after emergence just one seedling (young plant) was maintained to evaluate the growth. Depth of sowing was 1 cm below soil surface and they were moistened daily. Emergence was evaluated nine days after sowing. Seedlings were analyzed as to length of roots and shoots (LR and LS) (cm), dry matter of roots and shoots (DMR and DMS) (g), number of leaves (NL), diameter of stem (DS) (mm) and total dry matter (TDM) (g) 24 days after sowing. Dry matter measurements were achieved putting the samples in an oven with forced ventilation set at 60 ºC till constant weight (BRASIL, 2009).

***Experimental design and Statistical analysis*:** Germination assays were completely randomized with 4 replicates x 25 seeds. Germination and seedling data expressed in percentage were transformed using arc sine$\sqrt{x}/100$ function. Accelerated ageing was analyzed in factorial scheme with 2 treatments (osmoprimed and unprimed seeds) x 2 periods of ageing (48 h and 96 h). Cross tolerance was also analyzed in factorial scheme with 2 treatments (osmoprimed and unprimed seeds) x 3 temperatures (25, 37 and 40 ºC). The last assay in greenhouse was also completely randomized with 4 replicates x 10 pots x 2 treatments (osmoprimed and unprimed seeds). A statistical analysis was made by comparing means using Tukey Test at 5% probability through the statistical package SISVAR 4.3 (FERREIRA, 1999).

RESULTS AND DISCUSSION

Osmopriming of catingueira seeds

The seed moisture content was calculated before to perform the proposed assay. The average value was 5.38%, confirming previous data (ANTUNES et al., 2010) whose catingueira seeds have an orthodox behavior to desiccation tolerance during the storage. After that it was checked the effect of priming on seed vigour through some germination parameters (G_{max}, t_{50} and U_{8416}).

Final maximal germination (G_{max}) (figure 1) was improved in osmoprimed seeds (100%) and was significantly higher than in unprimed ones (94%). The explanation for that is germination advancement (HEYDECKER & COOLBEAR, 1977) based on metabolic activities before radicle emergence, such as: DNA repair, DNA replication and mobilization of storage proteins (POWELL et al., 2000; CHEN et al., 2010). The pattern of

water uptake during priming (slow and controlled) is also crucial to improve final germination (VARIER et al., 2010).

Jeller & Perez (2003a) observed an improvement in *Cassia excelsa* seed germination under stress conditions after priming (PEG); Jeller et al. (2003b) also observed beneficial effects of priming (PEG at -0.2 MPa) on seed germination of *Cassia excelsa* without drying and Kissman et al. (2010) verified increase in seed germination of *Stryphnodendron adstringens* and *Stryphnodendron polyphyllum* after priming (PEG at -0.5 MPa).

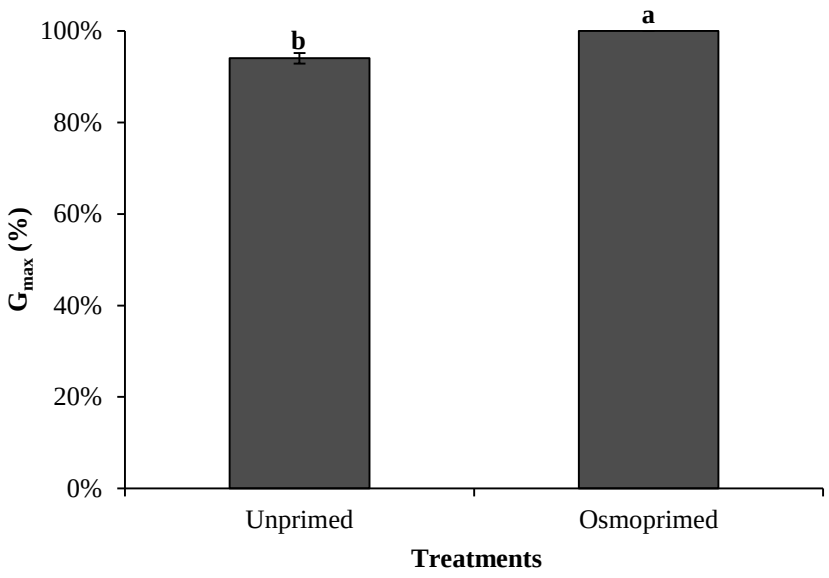

Figure 1. Final maximal germination (G_{max} %) of unprimed and osmoprimed *Poincianella pyramidalis* seeds. Averages with the same letter do not significantly differ by the Tukey test at 5% probability.

Priming had a significant effect ($p<0.05$) on rate (t_{50}) and uniformity (U_{8416}) of germination (figure 2A and 2B), with better results in osmoprimed seeds (t_{50} – 31.0 h and U_{8416} – 12.9 h) than in unprimed seeds (t_{50} – 61.2 h and U_{8416} – 48.7 h). By delaying germination, priming provides the additional repair time to the seeds and they could recover the capacity to germinate under standard conditions and indeed survive a further period of storage (BUTLER et al., 2009). PEG effectively regulates entry of water into the seed (without causing injury in leguminous seeds) and advances germination reactions (synchronization and germination rate) to a more or less fixed level determined by the water potential (-1.2 MPa in our case) (BASU et al., 1994; GHIYASI et al., 2008).

Enhanced germination speed after priming has been reported for forest species: Suñe et al. (2002) observed significant increase in germination speed and uniformity of *Adesmia latifolia* seeds conditioned in PEG; Priming of *Stryphnodendron adstringens* and *S. polyphyllum* seeds at -0.5MPa resulted in higher germination speed index (KISSMAN et al., 2010); *Cnidosculus juercifolius* seeds with low vigour conditioned in PEG showed higher germination speed after priming (SILVA et al., 2005); Adams (1999) observed significant improvement in germination speed after osmopriming in *Callitris verrucosa* and *C. preissii* seeds (natural from semi-arid region). Improvements in germination performance in crop species induced by priming has been also reported (ELKOKA et al., 2007; FAROOQ et al., 2005; SOEDA et al., 2005; NASCIMENTO et al., 2001).

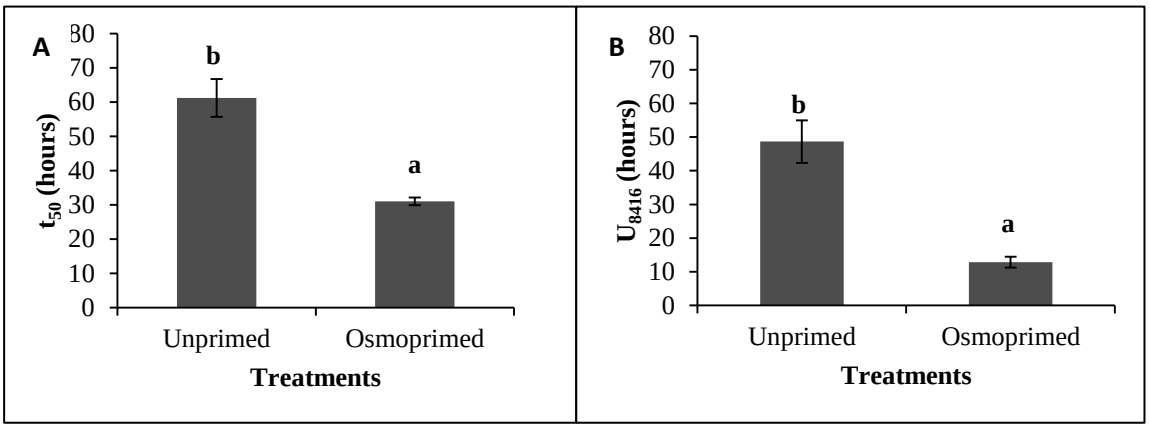

Figure 2. Germination parameters of unprimed and osmoprimed *Poincianella pyramidalis* seeds. A – Germination rate (t_{50} - hours); B – Germination uniformity (U_{8416} - hours). Averages with the same letter do not significantly differ by the Tukey´s test at 5% probability.

Effect of accelerated ageing in osmoprimed seeds

At 40 ºC temperature (T40 ºC) the interaction between treatments (unprimed and osmoprimed seeds) and ageing periods (48 and 96 hours) does not influenced final germination percentage ($p>0.05$) (Table 1) but interfered ($p<0.05$) in germination kinetics parameters of catingueira seeds (MTG and GSI).

There was no significant differences in final germination (%) among unaged seeds and seeds aged for 48 or 96 hours in both treatments proposed (osmoprimed and unprimed), although 96 hours of ageing seemed to improve little bit this parameter (96%) (Table 1). This suggests that catingueira seeds have high initial vigour and in those

conditions they can develop some adjustments to keep up metabolic reactions and enzymatic activities even under ageing (T40 ºC + 96% RH).

Moisture content increased with accelerated ageing of seeds. Unaged seeds presented 5.38%, unprimed seeds aged for 48 and 96 hours at 40 ºC showed respectively 22.00% and 26.38% and primed seeds 23.06% after 48 hours of ageing and 27.50% after 96 hours. The observed increase in moisture content by the seeds was not able to reduce final germination percentage, once these values were higher (table 1). In this way we could affirm that the ageing periods chosen at 40 ºC temperature were shorter to compromise seed vigour and longevity.

Corte et al. (2010) also observed that *Melanoxylon brauna* seeds kept high final germination after 72 hours of accelerated ageing (T40 ºC), starting decline just after 96 hours of ageing. Contradicting our findings Pinho et al. (2010) observed reduction in *Anadenanthera peregrina* seed germination already after 24 hours of accelerated ageing at T40 ºC been priming unable to revert the ageing effects on the seeds. Garcia et al. (2004) verified decrease in germination of *Anadenanthera colubrina* seeds after 24 hours of accelerated ageing at T40 ºC. Fanti & Perez (1999) also found reduction in seed germination of *Adenanthera pavonina* submitted to accelerated ageing using the same temperature.

Table 1. Germination percentage (G%) of *Poincianella pyramidalis* seeds submitted to accelerated ageing (AA) at T40 ºC.

Treatment	**Period ***			
	0h	**48h**	**96h**	**Average**
Unaged	91.0	/	/	91.0A
Unprimed	/	93.0	96.0	94.5A
Osmoprimed	/	91.0	96.0	93.5A
Average	91.0a	92.0a	96.0a	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

The kinetic parameter MTG evaluated at T40 ºC was significantly influenced by ageing time and osmopriming, since primed seeds showed a tendency to accelerate germination not only after 48 hours of ageing as well as after 96 hours (Table 2). Probably osmopriming provided to the seeds the additional time necessary to repair possible damages to DNA, to improve ATP levels and mRNA content and activate essential repair

enzymes culminating in more rapid germination even in accelerated ageing conditions (McDONALD, 1999).

Saha et al. (1990) found greater performance of germination (rapid and uniform germination) in primed *Glycine max* seeds than in unprimed seeds under ageing. The authors attribute their behavior to the increased amylase and dehydrogenase activity and lower lipid peroxidation. The invigoration treatment may act as a preventive measure against ageing, at least partly scavenging free radical formed during accelerated ageing (SHELAR, 2007).

Table 2. Mean Time of Germination (MTG - hours) of *Poincianella pyramidalis* seeds submitted to accelerated ageing (AA) at T40 ºC.

Treatment	**Period ***			
	0h	**48h**	**96h**	**Average**
Unaged	2.92	/	/	2.92
Unprimed	/	2.16bB	1.45aA	1.80
Osmoprimed	/	1.61aA	1.34aA	1.47
Average	2.92	1.88	1.40	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

Germination speed index of catingueira seeds in accelerated ageing conditions at T40 ºC was improved by osmopriming, although significant differences was observed just after 48 hours of ageing. Furthermore osmoprimed and unprimed seeds showed better rate of germination (speed) after 96 hours of accelerated ageing (Table 3), confirming the idea that catingueira seeds develop strategies to maintain germination in stress situation as accelerated ageing. Pinho et al. (2010) however found decline in germination speed of *A. peregrina* already after 24 hours of accelerated ageing (T40 ºC) even after priming. Braccini et al. (1999) also observed reduction in germination speed of *Glycine max* seeds (different lots) after 72 hours of ageing at 41 ºC temperature.

Table 3. Germination Speed Index (GSI) of *Poincianella pyramidalis* seeds submitted to accelerated ageing (AA) at T40 ºC.

Treatment	Period *			
	0h	**48h**	**96h**	**Average**
Unaged	9.04	/	/	9.04
Unprimed	/	13.37bB	19.08aA	16.23
Osmoprimed	/	17.87aA	19.88aA	18.88
Average	9.04	15.62	19.48	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

Under 45 ºC temperature (T45 ºC) it was observed a significant interaction ($p<0.05$) between the proposed treatments (osmoprimed and unprimed seeds) and ageing periods (48 and 96 hours), since there was a decrease in germination parameters (G%, MTG and GSI) as longer was the ageing period associated with priming treatment.

Final germination (G%) of unprimed and osmoprimed seeds 48 hours aged (88 and 67% respectively) was better than G% of seeds 96 hours aged (62 and 5%) (table 4) showing that longer ageing at high temperature (T45 ºC) and high relative humidity (92%) compromise viability and longevity of catingueira seeds. Osmoprimed seeds 96 hours aged (table 4) were more sensitive to the combination of osmopriming, ageing, high temperature and high relative humidity because those stress factors not only decreased drastically germination percentage as well triggered a high level of seed deterioration (data not showed). The explanation for that is a powerful oxidative stress generated (ROS accumulation) which probably damages proteins, nucleic acids, antioxidative enzymes (ascorbate peroxidase, catalase, superoxide dismutase, etc), disrupts endomembranes by lipid peroxidation and promotes some mitochondrial dysfunctions (decreasing ATP supply) (BAILY, 2004).

Moisture content of unaged seeds was 5.38%, unprimed seeds kept almost the same level in the two ageing periods 26.45% (48 hours) and 26.72% (96 hours) and osmoprimed seeds presented an increase of moisture content from 48 hours of ageing to 96 hours (32.72% and 34.04% respectively). These results are especially correlated to final germination data of osmoprimed seeds, aged for 96 hours, since those seeds showed decrease of germination probably due to increase of moisture content.

Table 4. Final germination (G%) of *Poincianella pyramidalis* seeds submitted to accelerated ageing (AA) at T45 ºC.

Treatment	Period *			
	0h	**48h**	**96h**	**Average**
Unaged	97	/	/	97
Unprimed	/	88aA	62bA	75
Osmoprimed	/	67aA	5bB	36
Average	97	77.5	33.5	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

Paiva et al. (2008) working with 3 different seed lots of *Macrotyloma axillare* aged for 48, 72 and 96 hours observed reduction on seed germination percentage when temperature was increased to 45ºC. Corte et al. (2010) studying the behavior of *Melanoxylon brauna* seeds under accelerated ageing at T45 ºC found a decline in germination especially after 96 hours of ageing. Oliveira et al. (2010) analyzing germination under accelerated ageing (at T43 ºC) of osmoprimed and unprimed *Sorghum bicolor* seeds found a negative correlation between priming and ageing.

Unprimed seeds required approximately 2.5 days in average to germinate. Those seeds increased time to germinate as ageing period was longer, but without significant differences between 48 and 96 hours of ageing (Table 5). The delay in germination of aged but still viable seeds might correspond to the time necessary for the cells re-initiate the antioxidant machinery, trying to escape from an oxidative stress (BAILLY et al., 1998; 2004). Osmoprimed seeds germinated faster after 96 hours of ageing, but this is related to the small number of vigorous seeds (Tables 4 and 5) that could germinate on that condition.

Table 5. Mean Time of Germination (MTG - hours) of *Poincianella pyramidalis* seeds submitted to accelerated ageing (AA) at T45 ºC.

Treatment	Period *			
	0h	**48h**	**96h**	**Average**
Unaged	2.45	/	/	2.45
Unprimed	/	1.80aA	2.54aB	2.17
Osmoprimed	/	2.58bA	1.58aA	2.08
Average	2.45	2.19	2.06	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

Germination speed index was reduced in osmoprimed seeds in both accelerated ageing periods, suggesting that osmopriming had a negative effect on seed vigour, probably compromising key enzymes responsible by the germinative process. Unprimed seeds aged for 48 hours however, kept higher rates even better than unaged seeds (Table 6).

Fanti & Perez (2003) studying primed seeds of *Chorisia speciosa* submitted to accelerated ageing at T45 ºC found an abrupt reduction in speed germination already after 48 hours of ageing. Oliveira et al. (2010) evaluating germination speed of primed and unprimed *S. bicolor* seeds under accelerated ageing (T43 °C) did not find significant differences among them.

Table 6. Germination Speed Index (GSI) of *Poincianella pyramidalis* seeds submitted to accelerated ageing (AA) at T45 ºC.

Treatment	**Period ***			
	0h	**48h**	**96h**	**Average**
Unaged	11.43	/	/	11.43
Unprimed	/	16.68aA	6.68bA	11.68
Osmoprimed	/	7.53aB	0.67bB	4.10
Average	11.43	12.10	3.67	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

Natural ageing of primed seeds

Osmoprimed and unprimed catingueira seeds two years stored in the fridge (8 ºC ±2 temperature) and packed in plastic bags showed a great germination performance keeping percentage and kinetic parameters higher as in newly collected seeds. Those conditions (temperature and package) already indicated by Antunes et al. (2010) to storage catingueira for one year really maintain viability and vigour of the seeds for longer time and to osmoprime seeds before storage seem to be an important tool to improve catingueira seed quality.

There was no statistical differences in final germination (G%) among the three seed lots analyzed showing that unprimed and osmoprimed seeds stored are able to keep the initial quality. Osmoprimed and newly collected seeds presented shorter MTG and higher GSI than unprimed ones (Table 7).

Table 7. Final Germination (G%), Mean time of germination (MTG) and Germination speed index (GSI) of fresh unprimed and osmoprimed *Poincianella pyramidalis* seeds stored for two years in the fridge and packed in plastic bags and newly collected seeds.

SEED LOT	G%	MTG	GSI
Newly collected seeds	98a	2.01a	15.13a
Unprimed seeds stored	94a	2.43b	11.25b
Osmoprimed seeds stored	98a	1.77a	17.57a

* Averages with the same letter do not significantly differ by the Tukey´s test at 5% probability.

Our results contradict most of literature that affirms priming impacting longevity in long term storage (HEYDECKER & GIBBINS, 1978; WALTERS, 1998). The effect of osmopriming, particularly with respect to subsequent longevity, can be influenced by the conditions immediately after priming. Thus if seeds are slowly dried after priming it is possible improvement in life span because they get enough time to proceed repair processes and also synthesize protectant proteins, as Late abundant embryogenesis proteins - LEAs (BUTLER et al., 2009; GURUSINGHE & BRADFORD, 2001; GURUSINGHE et al., 2002).

Bruggink et al. (1999) trying to restore longevity in pansy seeds submitted them to priming with subsequent slow or fast drying treatment and found greater results in primed pansy seeds slowly dried after 23 months of storage. Powell et al. (2000) however observed that the effect of aerated hydration of *Brassica oleracea* seeds with high initial vigour was decrease in germinability along storage.

Some authors believe in a correlation between natural and accelerated ageing, proposing that mechanisms that promote seed deterioration are the same in both situations (SANTOS & PAULA, 2007) while others affirm that accelerated ageing does not reproduce in a reliable way the metabolic events that happen during storage (FANAN et al., 2006; BAILLY et al., 1996). Based in our findings we could not affirm that seeds aged in the same way by the both used methods, since two years of storage kept seed vigour and viability while accelerated ageing for 96 hours at T45 ºC compromised drastically seed quality (osmoprimed seeds) (Tables 6 and 7).

Cross tolerance in primed seeds

The initial exposure of the seeds to the osmopriming, aiming higher performance in stressful temperatures (T37 ºC and T40 ºC), does not influenced final germination and

germination speed (Table 8). Thus, we could not affirm that there was a cross tolerance phenomenon induced by osmopriming. Probably the combination between high temperatures and osmopriming (PEG -1.2 MPa) was over than catingueira seeds could tolerate. In *Pterogyne nitens* seeds, priming (PEG+KNO_3) did not revert the negative effect of high temperature on seed germination (TONIN et al., 2005). Meanwhile, literature points out some reports attesting effectiveness of priming overcoming thermoinhibition in crop species. For example, osmopriming with PEG (-1.2 MPa) at 15 ºC temperature can overcome inhibitory effects of high temperature in thermosensitive lettuce seeds by weakening endosperm and increase of respiration and ATP production (NASCIMENTO et al., 2001; SMALL et al., 1993).

Table 8. Final Germination (G%) and Germination speed index (GSI) of osmoprimed and unprimed *Poincianella pyramidalis* seeds submitted to different temperatures. **T**=treatment; **U**=unprimed; **O=**osmoprimed; **A**=average.

T	**Temperature ºC ***							
	G%				**GSI**			
	25	**37**	**40**	**Average**	**25**	**37**	**40**	**Average**
U	90.0aA	46.0bA	25.0cA	53.67	10.54aA	2.56bA	1.04cA	4.71
O	89.0aA	38.0bA	19.0bA	48.67	9.59aA	1.84bA	1.06bA	4.16
A	89.5	42.0	22.0		10.06	2.20	1.05	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

Osmopriming also did not increase osmotic stress tolerance in catingueira seeds since it was not observed statistical differences in G% and GSI that confirms higher performance of osmoprimed seeds. The two kind of seeds (osmoprimed and unprimed) kept germination until -0.8 MPa and did not germinate at -1.2 MPa (Table 9). Once again the association between priming and stressful osmotic conditions compromised germination process in catingueira seeds. But for crops species as *Daucus carota*, osmopriming with aerated PEG solution provided improvement in seed germination under osmotic stress conditions (-0.4 MPa) (PEREIRA et al., 2009).

Table 9. Germination percentage (G%) and Germination speed index (GSI) of osmoprimed and unprimed *Poincianella pyramidalis* seeds submitted to different osmotic potentials (-MPa) of PEG solutions. **T**=treatment; **U**=unprimed; **O=**osmoprimed; **A**=average.

T	**Osmotic potential (-MPa) ***							
	G%				**GSI**			
	0.0	**0.8**	**1.2**	**Average**	**0.0**	**0.8**	**1.2**	**Average**
U	90.0aA	68.0bA	0.0cA	52.67	10.54aA	4.72bA	0.00cA	5.09
O	89.0aA	71.0bA	0.0cA	53.33	9.59aA	4.17bA	0.00cA	4.59
A	89.5	69.5	0.0		10.06	4.45	0.00	

*Averages with the same capital letter in the column and the same lower case letter in the line do not significantly differ by the Tukey´s test at 5% probability.

Seedling growth analysis from primed seeds

Seedlings obtained from osmoprimed and unprimed seeds and grown in greenhouse did not show significant differences among most of the vigour parameters evaluated in this assay (Table 10). The expected faster emergence in osmoprimed seedlings was not observed, as the one observed in osmoprimed seeds kept in lab conditions (Figures 1, 2A and 2B). Unprimed seedlings presented higher length and dry matter of root than osmoprimed seedlings (Table 10). Based in those results we can affirm that priming was not effective to promote improvements in seedling quality and also, since the seed lot had high initial vigour it was unnecessary to prime seeds before to sow them in field conditions. Perhaps using a seed lot with recognized low vigour the parameters evaluated would be better after osmopriming.

Wanli et al. (2001) studying the performance of hydro and osmoprimed *Peltophorum dubium* seedlings against unprimed seedlings found no significant differences for emergence percentage. Dry matter of shoot part was higher in unprimed seedlings than in primed seedlings (hydro and osmoprimed). Probably they also used a seed lot with high initial vigour, since those findings are very similar to catingueira results. Ghassemi-Golezani et al. (2008) also did not observed differences in emergence (%) among *Lens culinaris* seedlings coming from osmoprimed (PEG) and unprimed seeds. But when they tested the effect of hydropriming in emergence, hydroprimed seedlings got better results than unprimed ones. Suñé et al. (2002) obtained greater results in *Adesmia latifolia* seedlings coming from osmoprimed seeds, showing that emergence (%), seedling height and dry matter of seedlings were maximized by osmopriming (PEG).

Table 10. Vigour parameters of *Poincianella pyramidalis* seedlings grown at greenhouse coming from osmoprimed and unprimed seeds. **E% =** emergence percentage; **LR** = Length of root; **LS** = Length of shoot; **DMR =** Dry matter of root; **DMS** = Dry matter of shoot; **NL** = Number of leaves; **DS** = Diameter of stem; **TDM** = Total dry matter.

Treatments	E%	LR	LS	DMR	DMS	NL	DS	TDM
Osmoprimed	74.40a	9.25b	10.75a	0.010b	0.088a	2.00a	1.52a	0.098a
Unprimed	78.10a	13.12a	10.59a	0.015a	0.087a	2.05a	1.75a	0.103a

* Averages with the same letter do not significantly differ by the Tukey´s test at 5% probability.

CONCLUSIONS

- Osmopriming improves catingueira seed vigour and quality.
- Accelerated ageing performed at T40 ºC might be reverted by osmopriming, but at T45 ºC promotes deleterious effects on the seeds.
- Osmopriming did not compromise longevity of catingueira seeds stored in suitable conditions (plastic bags and fridge) since they had high initial vigour.
- Effects from too stressful conditions (as severe osmotic stress and high temperatures) could not be reverted by osmopriming in catingueira seeds.
- Osmopriming did not increase catingueira seedling performance in the greenhouse. Probably using seed lots with low quality, osmopriming works better.

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CHAPTER 3

DIFFERENTIALLY EXPRESSED GENES IN *Poincianella pyramidalis* (TUL.) L. P. QUEIROZ SEEDS UNDER OSMOPRIMING

Article to be submitted to Annals of Botany

Abstract: (**Differentially expressed genes in** *Poincianella pyramidalis* (**Tul.**) **L. P. Queiroz seeds under osmopriming)**. Osmopriming of seeds leads to changes in the expression of a large number of genes, since the osmotic agent used in the process (e.g. polyethyleneglycol) can reactivate metabolism although germination will be prevented. Priming treatments can increase seed vigour, allowing faster germination and field emergence, especially under adverse field conditions. In order to identify and characterize genes that are involved in the vigour improvement of *Poincianella pyramidalis* (catingueira) seeds upon priming, we produced two different Suppression Subtractive Hybridization (SSH) cDNA libraries. These were a Forward and Reverse subtraction of cDNA samples isolated from untreated dry seeds (unprimed) and osmoprimed seeds. The two different libraries were sequenced by Illumina GAII next generation sequencing, resulting in almost 20 million reads of 75 bp that could be assigned in 5,298 different genes. Of these 999 were only found in the unprimed seeds library and 2711 genes were specific for the osmoprimed seeds library. The functional annotation of these genes was analyzed and the differential expression of some genes from each library was confirmed by qRT-PCR analysis. Those genes might play an important role in vigour improvement upon priming and may be potential markers for tolerance to water stress in *P. pyramidalis* seeds.
Key-words: Water deficit, Catingueira, SSH, Next generation sequencing, gene expression.

Resumo: (**Genes diferencialmente expressos em sementes de** *Poincianella pyramidalis* (**Tul.**) **L. P. Queiroz submetidas ao osmocondicionamento**). O osmocondicionamento de sementes leva a modificações na expressão de um grande número de genes, uma vez que o agente osmótico utilizado no processo (como o polietilenoglicol) pode reativar o metabolismo, prevenindo a germinação. O osmocondicionamento pode aumentar o vigor das sementes, permitindo rápida germinação e emergência em campo, especialmente em condições naturais adversas. Visando identificar e caracterizar genes que estão envolvidos no aumento do vigor de sementes de *Poincianella pyramidalis* (catingueira) sob osmocondicionamento, foram produzidas duas bibliotecas de cDNA utilizando-se da técnica de Hibridização supressiva subtrativa (SSH). Estas corresponderam a uma subtração Direta e Reversa de amostras de cDNA isoladas a partir de sementes secas não tratadas (não osmocondicionadas) e a partir de sementes osmocondicionadas. As duas diferentes bibliotecas foram sequenciadas pela plataforma da *Illumina GAII next generation sequencing* resultando em quase 20 milhões de sequências de 75pb que foram montadas em 5,298 diferentes genes. Destes, 999 foram exclusivamente encontrados na biblioteca de sementes não osmocondicionadas e 2711 genes foram específicos da biblioteca de sementes osmocondicionadas. A anotação funcional destes genes foi analisada e a expressão diferencial de alguns genes a partir de cada biblioteca foi confirmada por análise de qRT-PCR. Os referidos genes podem desempenhar um papel importante no aumento do vigor sob condição de osmocondicionamento e funcionarem como marcadores de tolerância ao estresse hídrico em sementes de *P. pyramidalis.*
Palavras-chave: Déficit hídrico, Catingueira, SSH, *Next generation sequencing*, Expressão gênica.

INTRODUCTION

Priming is a commercially used technique for improving seed germination and seedling emergence. It involves imbibition of seeds in water (hydropriming), or in an osmoticum (e.g. polyethyleneglycol - PEG) (osmopriming), under controlled conditions to initiate early events of germination prior to radicle protrusion. Subsequently seeds are dried back to their initial moisture content (VARIER et al., 2010). After priming, seeds usually get faster and synchronized germination (HEYDECKER et al., 1978; CHEN et al., 2012). Better germination performance after priming has been reported in several species as in soybean (*Glycine max*), pepper (*Capsicum annuum*), spinach (*Spinacia oleracea*) and barba-timão (*Stryphnodendron adstringens*) (ZHUO et al., 2009; KORKMAZ & KORKMAZ, 2009; CHEN et al., 2010; KISSMAN et al., 2010).

It is well known the effects of PEG in seed germination, reducing water availability (CORTEZ-BAHEZA et al., 2007). Thus, genes induced during water deficit conditions could be related to osmopriming. They are thought to function (1) encoding products that protect plant cells against stresses such as heat shock proteins (HSPs), LEA proteins, osmoprotectants, antifreeze proteins, detoxification enzymes, free-radical scavengers, water transporters (aquaporins) and ion transporters (BLUMWALD et al., 2000; BRAY et al., 2000; WANG et al., 2000) and (2) activating group 1 through signaling cascades and transcriptional control, such as MAPK, CDPK, SOS kinase, phospholipases and transcriptional factors (SHINOZAKI et al., 2000; CHOI et al., 2000; FRANK et al., 2000; ZHU et al., 2001; RUDRABHATLA et al., 2002; SHINOZAKI et al., 2003; LUDWIG et al., 2004).

Nowadays molecular research to elucidate gene expression involved in priming treatments have been increased, but even though little information is available for crop species and even less for forest ones. Soeda et al. (2005) working with osmoprimed (PEG) *Brassica oleracea* seeds found some genes with increased expression during priming and also during germination (S-Adenosyl-Met synthetase, Sugar epimerase, Dioxygenase, Glutathione tranferase, Stress related proteins, L-Ascorbate peroxidase). Those genes mostly code for proteins involved in energy metabolism and chemical defence mechanisms. Cortez-Baheza et al. (2007) studying osmoprimed (PEG+GA_3) *Capsicum annuum* seeds found some transcripts related to LEA proteins, Heat Shock proteins, Proteinase inhibitors, Replication and Signal transduction; Schwember & Bradford (2010) evaluating the expression of *LsNCED4*, *LsGA3ox1* and *LsACS1* in relation to the effect of

priming, on the maximum temperature for germination of *Lactuca sativa* seeds, found that priming stimulated suppression of *LsNCED4* expression and enhancement of *LsGA3ox1* and *LsACS1* expression, alleviating thermoinhibition in lettuce seeds.

Since priming alters the physiological state of seeds at transcriptional level (CORTEZ-BAHEZA et al., 2007), Suppressive Subtractive Hybridization (SSH) might be an important tool to compare two populations of mRNA and obtain clones of genes that are expressed in one population (tester) but not in the other (driver). This is possible thanks to normalization steps and rounds of PCR amplification which allow differential gene expression of transcripts related just to the target situation (DIATCHENKO et al., 1996; LUKYANOV et al., 2007). The technique has been widely used to find out genes in response to water stress: *Medicago truncatula* (BOUTON et al., 2005), *Triticum aestivum* (WAY et al., 2005), *Setaria italica* (ZHANG et al., 2007), *Glycine max* (BEILINSON et al., 2005; CLEMENT et al., 2008; RODRIGUES et al., 2012) and also in response to osmopriming: *Capsicum annuum* (CORTEZ-BAHEZA et al., 2007; 2008).

Considering that there are few reports in the literature about gene expression in response to osmopriming treatment in forest species, we proposed in this approach to identify and characterize genes that are involved in the vigour improvement of *Poincianella pyramidalis* (Tul.) L. P. Queiroz (catingueira) seeds upon osmopriming by SSH. The data generated by this proposal will provide basic information for others future molecular approaches involving Brazilian semi-arid tree species.

MATERIALS AND METHODS

***1. Seed collection*:** The seeds were harvested from *P. pyramidalis* trees, located at Petrolina – PE, Brazil, under the following coordinates: 9º 30' 21" S 40º 30' 21" W in September 2008. After harvest the seeds were dried in the shade at room temperature, during three days, aiming to reduce their moisture content.

***2. Osmopriming*:** Seeds of *P. pyramidalis* with 5.38% moisture content were osmoprimed in polyethyleneglycol 8000 (PEG 8000) at -1.2 MPa for seven days at 25 ºC with constant light. A roller system kept samples under agitation providing aeration to them. The osmotic potential was chosen based on the previous osmotic stress assay of *P. pyramidalis* seeds. On completion of treatment the seeds were removed and rinsed out in distilled water then dried for four days in an incubator at 20 ºC and 30% RH up to they reach their initial

moisture content (HEYDECKER & GIBBINS, 1978; POWELL et al., 2000; NASCIMENTO et al., 2001). Unprimed seeds (untreated dry seeds) were considered as control. It was used 3 biological replicates x 10 seeds per treatment.

3. Subtraction libraries generation

***3.1 Total RNA isolation*:** SV Total RNA Isolation System Kit (Promega, Madison, USA) was used to extract total RNA from 30 mg of unprimed and osmoprimed seeds. Integrity and fragment size of RNA samples from both treatments were analyzed by 1.5% agarose gel electrophoresis while concentration and purity were determined by $OD_{260/280 \text{ and } 260/230}$ values using the nanodrop.

3.2 cDNA synthesis: cDNA synthesis was performed from total RNA samples obtained from unprimed and osmoprimed seeds, making use of Super Smart ^{TM} PCR cDNA Synthesis Kit (Clontech Laboratories, Inc., Canada). Starting material was equals to 1 µg of total RNA for each sample and all centrifuge steps were proceeded at 4 ºC. It was performed an optimization of number of cycles before PCR which correspond to the optimal number of PCR cycles, before the samples reach the plateau phase (according manufacturer´s recommendations).

3.3 Subtraction: Two subtraction libraries were generated making use of PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories, Inc., Canada) (Figure 2, page 9). In the **forward library** cDNA from osmoprimed seeds was considered as "tester" and cDNA from unprimed seeds as "driver". In the **reverse library** cDNA from unprimed seeds was considered as "tester" and cDNA from osmoprimed seeds as "driver". The tester and driver cDNAs were digested with Rsa I, a four-base-cutting restriction enzyme that yields blunt ends. The tester cDNA was then subdivided into two portions, and each was ligated with a different cDNA adaptor (1 and 2R). The two adaptors had stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in. Two hybridizations were then performed. In the first, an excess of driver was added to each sample of tester. The samples were then heat denatured and allowed to anneal, generating the type **a**, **b**, **c**, and **d** molecules in each sample. The concentration of high- and low-abundance sequences was equalized among the type **a** molecules because reannealing is faster for the more abundant molecules. At the same time, type **a** molecules were

significantly enriched for differentially expressed sequences while cDNAs that were not differentially expressed formed type **c** molecules with the driver. During the second hybridization, the two primary hybridization samples were mixed together without denaturing. Thus, only the remaining equalized and subtracted ss tester cDNAs (single strand tester cDNAs) could be reassociated to form new type **e** hybrids. These new hybrids are ds tester (double strand tester) molecules with different ends, which correspond to the sequences of Adaptors 1 and 2R. Fresh denatured driver cDNA was added (again, without denaturing the subtraction mix) to further enrich fraction **e** for differentially expressed sequences. After filling in the ends by DNA polymerase, the type **e** molecules—the differentially expressed tester sequences—had different annealing sites for the nested primers on their 5' and 3' ends. The entire population of molecules was then subjected to PCR to amplify the desired differentially expressed sequences. During this PCR, type **a** and **d** molecules are missing primer annealing sites, and thus could not be amplified. Due to the suppression PCR effect, most type **b** molecules formed a pan-like structure that prevent their exponential amplification. Type **c** molecules had only one primer annealing site and amplified linearly. **Only type e molecules —** the equalized, differentially expressed sequences with two different adaptors **— amplified exponentially**. Finally, a secondary PCR amplification was performed using nested primers to further reduce any background PCR product and enrich for differentially expressed sequences. Digestion, adaptor ligation efficiency (performed with primer Actin *Medicago sativa*, which amplified in catingueira samples) (Table 1) and PCR amplification products were checked in agarose/EtBr gel electrophoresis. Subtraction efficiency was checked by qRT-PCR. Both subtraction libraries (forward and reverse) were submitted to sequencing.

4. Sequencing (RNA-seq) and Bioinformatics Analysis: Sequencing of mRNA from both libraries was performed by Illumina GAII next generation sequencing platform, according to the manufacturer's instructions. The reads of the two libraries were assembled into contigs using the De Novo Assembler Velvet v1.07 package. The assembly is a trade-off between the number of contigs, accuracy of the assembly, and the amount of data not included in the assembly. Assembled contigs were blasted against the Genbank database (www.ncbi.nlm.nih.gov/genbank) using Blast X for annotation and homology search. The two highest scoring hits were kept for annotation. The reads of the individual samples were aligned onto the assembled contigs using the Tophat v.1.0.13 package (TRAPNELL et al., 2009). The corresponding expression values of the contigs were expressed in FKPM

(Fragments per Kilobase of Exon per Million fragments mapped) using Cufflinks v0.8.2 (TRAPNELL et al., 2010). The differentially expressed sequences annotated of each library were categorized based on Gene Ontology (GO- http://www.geneontology.org/).

5. Gene expression assay by quantitative Reverse Transcription PCR (qRT-PCR): To validate RNA-seq results, the expression of eight genes (related to osmoprimed and unprimed libraries) (Table 1) was tested by qRT-PCR. Firstly total RNA was isolated from unprimed and osmoprimed seeds using Concert Plant RNA Reagent (Invitrogen, Carlsbad, USA) according the manufacturer´s instructions. After isolation total RNA samples were submitted to some phenol-chlorophorm steps and ethanol precipitation to clean up them. A DNAse treatment was performed making use of RQ1 DNAse enzyme (Promega, Madison, USA) followed by phenol-chlorophorm steps and ethanol precipitation again. cDNA was synthesized from total RNA using ImProm-II™ Reverse Transcription System Kit (Promega, Madison, USA). Primers for qRT-PCR (Table 1) were generated using IDT (Integrated DNA Technology) platform (http://www.idtdna.com/scitools/Applications/RealTimePCR) and ordered from Operon and IDT Companies. PCR reactions were composed by 10 µL of SYBR® Green PCR Master Mix 2X (Applied Biosystems, California, USA), 5 µl of cDNA, 0.5 µL of each 10 µM primer (forward and reverse) and 4 µL of ultrapure water totalizing a final volume of 20 µL. For negative control, ultrapure water was used as template in the PCR reaction. The reactions were performed in an Applied 7500 Fast Real Time PCR System (Applied Biosystems, California, USA). PCR conditions were: an initial step at 95 °C for 3 min, followed by 40 cycles with 15 s at 95 °C and 1 min at 60 °C. To verify specificity of amplification and absence of genomic DNA contamination, it was performed a melting curve analysis. Primer efficiency (acceptable ranging from 85 to 115%) was tested using a standard curve for each gene, based in serial dilutions (1, 2, 4, 8 and 16x). Based in some stable genes in *Arabidopsis thaliana* and *Lycopersicon esculentum* presented by Dekkers et al. (2012) we chose two reference genes (NODE 580 and NODE 45602) (Table 1), without differential expression in both libraries, to normalize the expression of the target genes. The stability of both genes was evaluated by geNorm program ($M \leq 0.5$) (HELLEMANS et al., 2007). Unprimed seeds were used as calibrator sample. Relative expression of each target gene was calculated by Pfaffl method (PFAFFL et al., 2001).

Experimental design and statistical analysis: Gene expression experiments were done with 3 biological replicates (10 seeds) x 2 technical replicates per treatment. Relative expression values of each treatment were transformed in $\log_2$ and compared by Test t ($p<0.05$) through SISVAR software (FERREIRA, 1999).

RESULTS

Construction of forward and reverse subtracted cDNA libraries

RNA isolation

Total RNA samples of unprimed and osmoprimed seeds were isolated prior to cDNA synthesis. Figure 1 and Table 2 present data about quality and concentration.

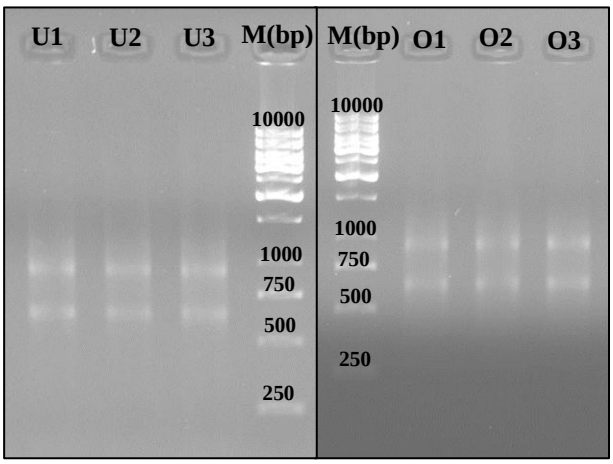

Figure 1. Total RNA samples from unprimed (U) and osmoprimed (O) *P. pyramidalis* seeds in 1.5% agarose gel.

Table 2. Concentration and quality ($A_{260/280}$ and $A_{260/230}$) of total RNA samples from unprimed (U) and osmoprimed (O) *P. pyramidalis* seeds.

Sample/Replicate	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
U1	497.6	2.22	2.22
U2	327.3	2.17	2.15
U3	292.6	2.19	2.06
O1	405.0	2.17	2.17
O2	300.2	2.18	2.05
O3	251.5	2.21	2.12

Table 1. Target genes, contig names (CA), gene-specific primer sequences, annealing temperature (AT), size of the fragments (SF) and primer efficiency (E).

Annotation	Contig name	Forward sequence	Reverse Sequence	AT	SF	E
Glycine max serine/threonine protein phosphatase 2A*	NODE 580	GCCAAGATGACATGCCTATG	AATCTTGAGGCTCCAACAG	60 ºC	198 bp	91%
Small GTP binding protein SAR 1A (SAR 2) *Arabidopsis thaliana**	NODE 45602	GGGCTAGATAATGCTGGAAAG	GCCTTGAATTTTATCTTCCCG	60 ºC	125 bp	94%
Glycine max seed maturation protein (GmPM3) mRNA	NODE 1142	TATCATTCCTCCACGCCCT	TCATCAACGGCCAAACCT	60 ºC	143 bp	97%
Oxidoreductase, putative [*Ricinus communis*]	NODE 46022	ACCAACATTAACCACAAGAGAG	TCGGACTGAAACCACATGC	60 ºC	139 bp	97%
Glycine max metallothionein-II protein (PGMPM19)	NODE 1074	CAGGAAGCACAGGAGCAA	AGATGTTCAAGTAGCGGGT	60 ºC	136 bp	110%
Ca+2-binding EF hand protein [*Glycine max*] >gi|255625919|gb|ACU13304.1| unknown [*Glycine max*]	NODE 86	GGGAGACTTATAGGGGGATG	CCTGTTTACGTTGTGGATGT	60 ºC	151 bp	87%
ATGSTU25 (Glutathione S-Transferase Tau 25)	NODE 37111	CAGAAGGAGAGTGTTTCCAAG	GCTTTATTCAGAGACAACCAAAC	60 ºC	169 bp	92%
Auxin-induced glutathione S transferase [*Prosopis juliflora*]	NODE 4738	CATGCATCTCTTAGCCCAAG	AGGGATTAAAGCAATTGGAGG	60 ºC	182 bp	110%
Tonoplast intrinsic protein alpha TIP [*Arachis hypogaea*]	NODE 753	GATTTACAGAGATGCAGGGTC	AGCGATCCAGTAATAGACGG	60 ºC	190 bp	94%
Glycine max seed maturation protein PM27 (PM27) mRNA	NODE 3870	AAACAGTGACAAGCGAGG	GGGTTATGGCTTATAGCGTTG	60 ºC	126 bp	97%
Actin *Medicago sativa***	-	TCCATCATGAAGTGCGATGT	AACCTCCGATCCAGACACTG	60 ºC	189 bp	-

*Reference genes chosen from stable genes presented by Dekkers et al. (2012).

** Primer used to test adaptor ligation efficiency in catingueira samples.

Total RNA samples showed high quality which was demonstrated by good integrity of bands, no genomic DNA contamination, proportion 1:1 among the bands coming from subunits 18S and 28S of rRNA and ratios $A_{260/280}$ and $A_{260/230}$ ranging from 1.7 to 2.0 (what means for the first ratio no contamination with proteins and for the second one, no contamination with polyphenols and polysaccharides) (SAMBROOK et al., 2000).

cDNA synthesis

cDNA samples were synthesized from 1μg of total RNA samples making use of Super Smart ^{TM} PCR cDNA Synthesis Kit, an optimized protocol which provides to RT enzyme transcribe entire mRNA sequence (including the 5' end of genes) (Figure 2).

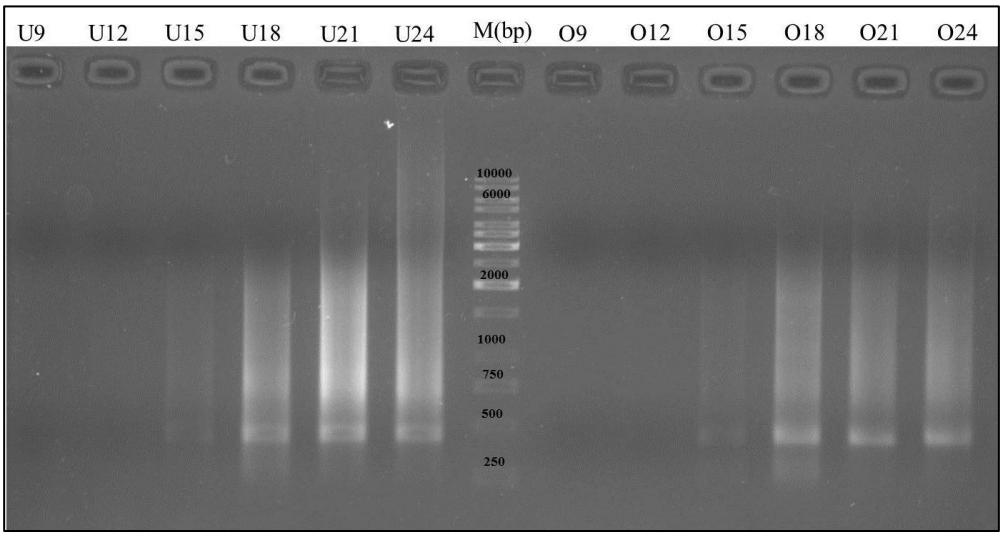

Figure 2. Optimization of PCR cycles number to generate double strand cDNA from total RNA samples in 1.2% agarose gel. U9 to U24 = Unprimed samples 9 to 24 PCR cycles. O9 to O24 = Osmoprimed samples 9 to 24 PCR cycles. Exponential phase = 18 cycles unprimed samples and 17 cycles for osmoprimed samples.

First strand was synthesized and after that an optimization of number of PCR cycles to generate double strand cDNA (*ds* cDNA) was performed. *ds* cDNA from total RNA of unprimed seeds was acquired after 1 min at 95 ºC and **18 cycles** of 10 sec at 95 ºC; 10 sec at 65 ºC; 6 min at 68 ºC. *ds* cDNA from total RNA of osmoprimed seeds was acquired by the same way, using **17 cycles** instead 18 (Figure 2).

Subtraction

To isolate differentially expressed genes induced by osmopriming, SSH was employed in the construction of subtracted cDNA library. Once we planned a differential screening of the subtracted library, it was performed subtractions in both directions: forward and reverse. The first step to produce them was Rsa I digestion, generating shorter, blunt-ended *ds* cDNA fragments, which are optimal for subtraction and required for adaptor ligation. The digestion with Rsa I was successfully achieved since the size distribution of unprimed (UA) and osmoprimed (OA) fragments was reduced (Figure 3).

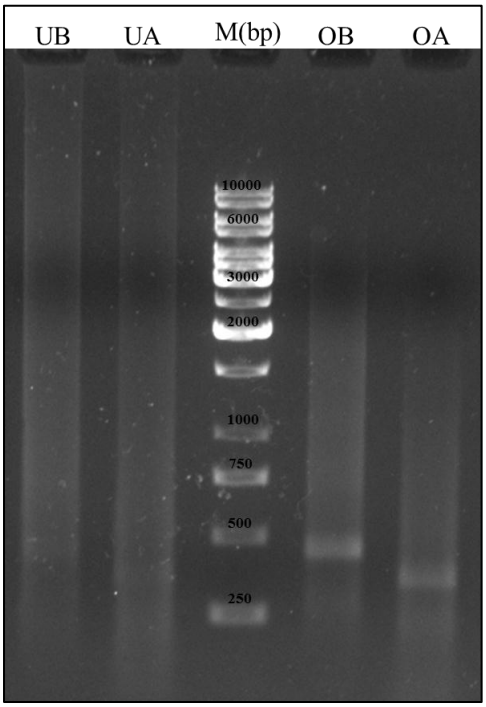

Figure 3. cDNA from unprimed and osmoprimed seeds before and after digestion with Rsa I. UB and UA = Unprimed seeds before and after digestion. OB and OA = Osmoprimed seeds before and after digestion.

After digestion each tester sample from the two libraries (forward = osmoprimed seeds and reverse = unprimed seeds) was divided in two portions to be bound to different adaptors (1 and 2R). An efficiency test prior to hybridization step was performed, to verify the ligation of adaptors in the different cDNA testers. Actin *Medicago sativa* primers (forward and reverse) (Table 1) and PCR Primer 1 (provided by PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories, Inc., Canada)) were used to proceed the test. We found amplification products with 200 bp from PCR

Primer 1 + Actin forward as well as amplification products with 189 bp from Actin forward + Actin reverse, in both cDNA testers. Since the bands coming from the amplified PCR products had the same intensity, we can affirm that at least 25% of the cDNA testers were ligated to the adaptors (1 and 2R) (according manufacturer´s recommendations) (Figure 4).

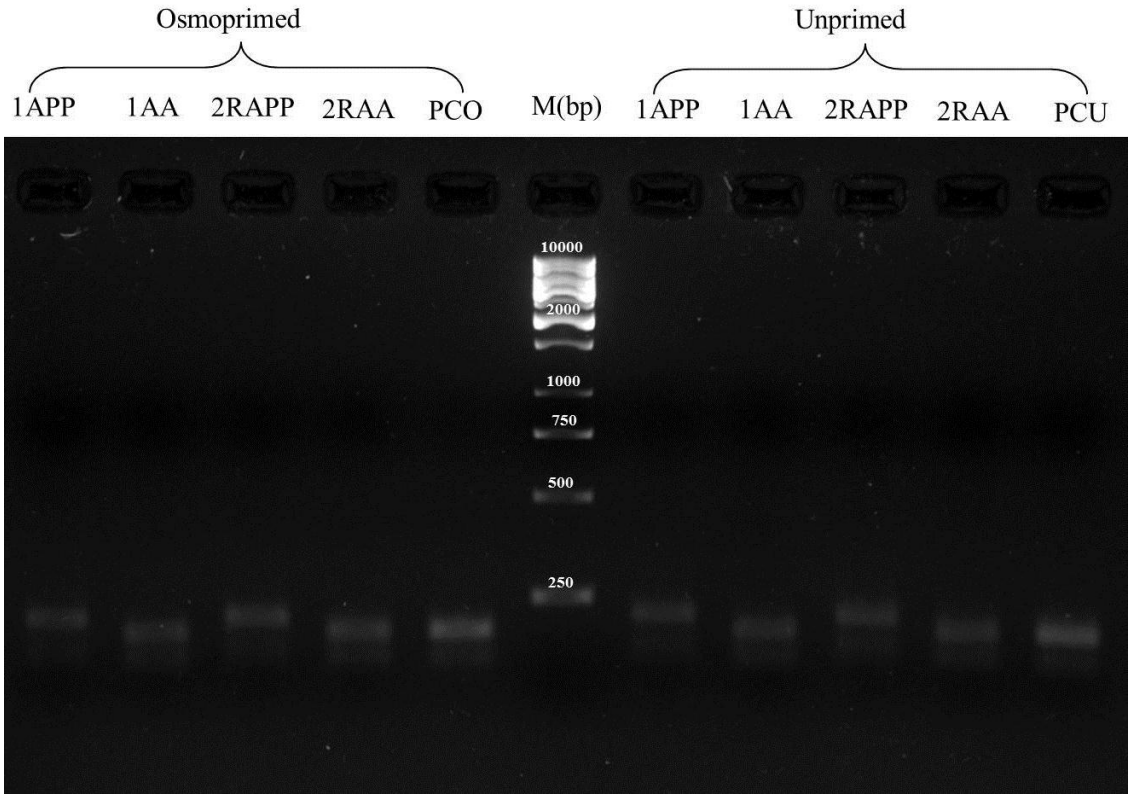

Figure 4. Adaptor ligated cDNA samples amplified with different primers in 2.0% agarose gel. 1APP = Adaptor 1 + Actin *Medicago sativa* forward + PCR Primer 1; 2RAPP = Adaptor 2R + Actin *M. sativa* forward + PCR Primer 1; 1AA = Adaptor 1 + Actin *M. sativa* forward + Actin *M. sativa* reverse; 2RAA = adaptor 2R + Actin *M. sativa* forward + Actin *M. sativa* reverse; PCO = Positive control osmoprimed (unsubtracted ligated to both adaptors and amplified by Actin *M. sativa* forward and reverse); PCU = Positive control unprimed (unsubtracted ligated to both adaptors and amplified by Actin *M. sativa* forward and reverse).

Adaptor ligated tester cDNA samples from both libraries (forward and reverse), were enriched for differentially expressed sequences, after two rounds of hybridization. In the first hybridization step those sequences were equalized and enriched and in the

second hybridization step, templates for PCR amplification were generated from the differentially expressed sequences.

The primary PCR amplification showed that in the subtracted libraries only ds cDNAs with different adaptors (1 and 2R) on each end were exponentially amplified. At this phase adaptors were extended creating sites for PCR primer 1, which was fundamental for amplification of differentially expressed sequences. The size distribution in the unsubtracted samples from each library was much higher than in subtracted ones (Figure 5).

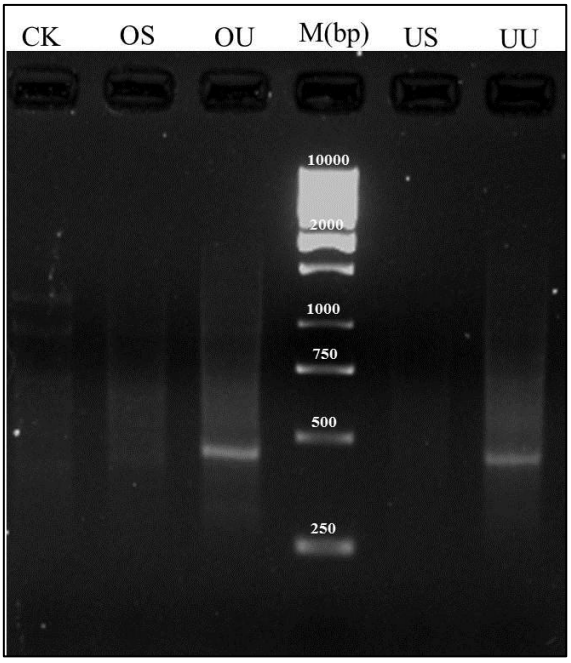

Figure 5. Primary PCR products from unsubtracted and subtracted cDNA samples in 2.0% agarose gel. Ck = PCR control subtracted cDNA (subtracted mixture of Hae III-digested ϕX174 DNA provided by the kit); OS = subtracted cDNA from osmoprimed seeds (Forward library); OU = unsubtracted cDNA from osmoprimed seeds (Forward library); US = subtracted cDNA from unprimed seeds (Reverse library); UU = unsubtracted cDNA unprimed seeds (Reverse library).

The secondary PCR amplification, making use of nested primers, reduced background PCR products and especially enriched each library for differentially expressed sequences. Size distribution in subtracted samples was drastically reduced, indicating a good signal of efficient subtraction, and also the pattern of bands in the forward library (osmoprimed seeds – target situation) was very similar to the one observed in the control from the Kit (Figure 6).

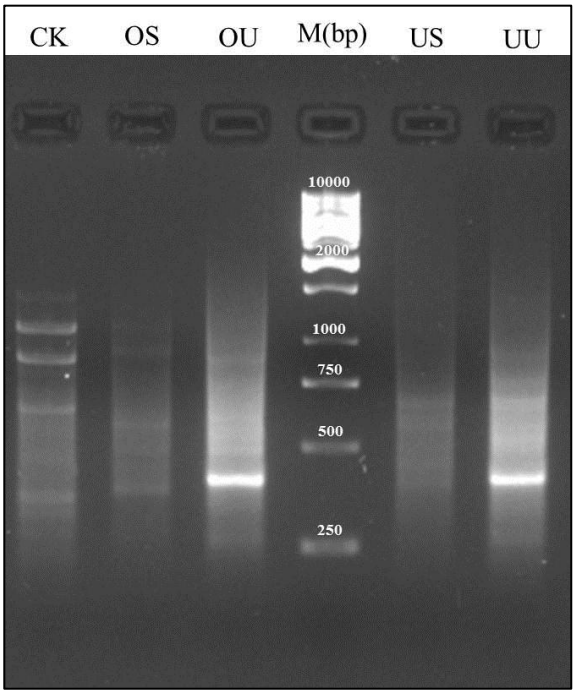

Figure 6. Secondary PCR products from unsubtracted and subtracted cDNA samples in 2.0% agarose gel. Ck = PCR control subtracted cDNA (subtracted mixture of Hae III-digested ϕX174 DNA provided by the kit); OS = subtracted cDNA from osmoprimed seeds (Forward library); OU = unsubtracted cDNA from osmoprimed seeds (Forward library); US = subtracted cDNA from unprimed seeds (Reverse library); UU = unsubtracted cDNA unprimed seeds (Reverse library).

At the end of the subtraction process, it was performed an efficiency test through a qRT-PCR analysis. It was used a Myiq Single Color Real Time PCR Detection System. The expected results were no PCR products or late PCR products for subtracted samples and early PCR products for unsubtracted ones, making use of *Actin Medicago sativa* primer. We found PCR products after 34 cycles in the subtracted cDNA forward library. We did not detect any PCR product in the subtracted cDNA reverse library. After 22 PCR cycles, we found products from the unsubtracted cDNA samples in both libraries. After subtraction, the cDNA libraries (forward and reverse) and the control provided by Clontech kit were analyzed as to concentration and quality. All of them presented high quality and concentration (Table 3).

Table 3. Concentration and quality (ratios $A_{260/280}$ and $A_{260/230}$) of subtracted cDNA libraries (forward = osmoprimed seeds and reverse = unprimed seeds).

Sample	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
Forward	508.3	1.88	1.86
Reverse	539.8	1.87	1.85
Control kit	539.8	1.87	1.87

Sequencing Data (RNA-seq)

Sequencing resulted in almost 20 million reads of 75 bp that could be assigned in 5,298 contigs. The average length of contigs was 355 bp and maximal length was 1,699 bp. Among the 5,298 contigs, 999 were only found in the unprimed especific library and 2711 genes were especific for the osmoprimed library. 1588 contigs were shared for both libraries.

The thirty most abundant genes in the especific osmoprimed library are presented in Table 4. Among them, some genes had interesting relation to metabolic functions and water deficit conditions: NODE 44560 (*Lotus corniculatus* var. japonicus mRNA for alcohol dehydrogenase); NODE 45006 (*Glycine max* NAC domain protein (NAC27) mRNA); NODE 43966 (p-hydroxyphenylpyruvate dioxygenase [*Mangifera indica*]) and NODE 44626 (*Arachis hypogaea* double-stranded DNA-binding protein mRNA).

The thirty most abundant genes in the especific unprimed library are presented in Table 5. Most of them are related to cellular maintenance in the dry stage, what is expected, since this library is composed by untreated dry seeds. Among the genes with recognized importance we can cite: NODE 753 (*Arachis hypogaea* tonoplast intrinsic protein alpha TIP mRNA); NODE 1471 (*Arachis hypogaea* desiccation protectant protein LEA 14 mRNA); NODE 3870 (Glycine max seed maturation protein PM27 (PM27) mRNA) and NODE 3732 (*Nicotiana tabacum* mRNA for dehydrin).

Table 4. The thirty most abundant genes in especific osmoprimed library.

Contig	Accession	Description (BLASTX)	E-value	FPKM
NODE_46224	gi|356549751|	PREDICTED: stem-specific protein TSJT1-like isoform 1 [*Glycine max*]	4,83E-17	20509,6
NODE_44560	emb|AJ717414.1|	***Lotus corniculatus* var. japonicus mRNA for alcohol dehydrogenase**	1E-59	12261
NODE_46047	gb|U02886.1|	*Atriplex nummularia* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA	8E-44	10230,8
NODE_44512	unknown	unknown	unknown	7790,72
NODE_43340	gi|145617261|	glyceraldehyde-3-phosphate dehydrogenase [*Medicago sativa*]	1,88E-33	7503,85
NODE_46673	gi|18397956|	maternal effect embryo arrest 14 protein [*Arabidopsis thaliana*]	4,07E-10	7318,8
NODE_44497	gb|BT092730.1|	Hypoxia induced protein conserved region containing protein expressed [*Oryza sativa* Japonica]	2,90E-07	6931,72
NODE_46535	gi|351728107|	uncharacterized protein [*Glycine max*]	9,60E-11	6623,02
NODE_44444	unknown	unknown	unknown	6423,44
NODE_46655	dbj|AK245638.1|	*Glycine max* cDNA	2E-19	6335,34
NODE_45006	gb|EU661924.1|	***Glycine max* NAC domain protein (NAC27) mRNA**	4E-13	5198,68
NODE_1217	gi|302539119|	predicted protein [*Streptomyces* sp. C]	2,57E-06	4896,35
NODE_46464	gi|77548353|gb|	Hypoxia induced protein conserved region containing protein expressed [*Oryza sativa* Japonica]	8,60E-05	4655,91
NODE_44547	gi|116871386|gb	xyloglucan endotransglycosylase 3 [*Litchi chinensis*]	8,44E-15	4594,97
NODE_31770	IPR005050	Early nodulin 93 ENOD93 protein	4,30E-21	4557,24
NODE_45062	gi|356572510	PREDICTED: pyruvate kinase, cytosolic isozyme-like [*Glycine max*]	9,87E-19	4533,71
NODE_46498	gi|380505034|	glyderaldehyde-3-phosphate dehydrogenase, partial [*Nicotiana benthamiana*]	8,14E-20	4347,03
NODE_43966	gi|309260073|	**p-hydroxyphenylpyruvate dioxygenase [*Mangifera indica*]**	6,43E-56	4329,38
NODE_44950	gb|BT092177.1|	Soybean unknown mRNA	8E-08	4292,19
NODE_45791	gi|356505332|	PREDICTED: fructose-bisphosphate aldolase, cytoplasmic isozyme [*Glycine max*]	1,68E-22	4206,16

Table 4. Continued

Contig	Accession	Description (BLASTX)	E-value	FPKM
NODE_40249	dbj|AP010367.1|	*Lotus japonicus* genomic DNA, chromosome 3	0,000007	4204,79
NODE_44626	gb|DQ889514.1|	***Arachis hypogaea* double-stranded DNA-binding protein mRNA**	1E-127	4135,34
NODE_45451	gi|357506055|	Pyruvate decarboxylase isozyme [*Medicago truncatula*]	1,31E-20	4090,7
NODE_45893	unknown	unknown	unknown	3859,8
NODE_43241	gi|357160930|	PREDICTED: pyruvate kinase, cytosolic isozyme-like [*Brachypodium distachyon*]	3,10E-41	3751,69
NODE_44164	gi|357452667|	Thioredoxin-like protein [*Medicago truncatula*]	7,50E-55	3678,84
NODE_46165	gb|BT092470.1|	Soybean unknown mRNA	0,0002	3386,19
NODE_44008	gb|BT093226.1|	Soybean unknown mRNA	0,000001	3369,25
NODE_45002	dbj|AP010309.1|	*Lotus japonicus* genomic DNA, chromosome 5	0,000001	3365,2
NODE_43340	gi|145617261|	glyceraldehyde-3-phosphate dehydrogenase [*Medicago sativa*]	1,88E-38	3308,86

Table 5. The thirty most abundant genes in especific unprimed library.

Contig	Accession	Description (BLASTX)	E-value	FPKM
NODE_3346	unknown	unknown	unknown	6002,5
NODE_4050	GQ997462.1	*Lonicera japonica* putative RF3 protein (ycf3) gene, chloroplast	1,00E-53	5918,46
NODE_33184	unknown	unknown	unknown	5181,2
NODE_590	gb|EU717241.1|	*Lespedeza cuneata* caseinolytic ATP-dependent protease (clpP) gene, chloroplast	4E-47	5129,92
NODE_4460	unknown	unknown	unknown	4638,69
NODE_2798	AAY34438.1	Kunitz-type chymotrypsin inhibitor [*Psophocarpus tetragonolobus*]	1,00E-12	4534,97
NODE_840	ref|XM_002517608.1|	*Ricinus communis* processing-splicing factor, putative, mRNA.	3E-60	4482,79
NODE_3807	gb|GQ998095.1|	*Cornus florida* cytochrome b6 protein (petB) gene, chloroplast.	2E-49	4393,27
NODE_753	gi|115187427|	**Tonoplast intrinsic protein alpha TIP [*Arachis hypogaea*]**	1,86E-46	4385,09
NODE_13392	unknown	unknown	unknown	4217,03
NODE_4048	gi|351720672|	Trypsin inhibitor p20 precursor [*Glycine max*]	2,70E-10	4125,24
NODE_1471	gb|DQ296034.1|	***Arachis hypogaea* desiccation protectant protein LEA 14 mRNA**	1E-23	4035,95
NODE_11565	unknown	unknown	unknown	3996,92
NODE_665	gb|EU811880.1|	*Vachellia bravoensis* AtpB (atpB) gene and atpB-rbcL intergenic spacer; chloroplast	1E-113	3966,16
NODE_3209	unknown	unknown	unknown	3907,22
NODE_3870	gb|AF117723.1|AF117723	***Glycine max* seed maturation protein PM27 (PM27) mRNA**	1E-35	3839,69
NODE_2365	AAC61881.1	11S storage globulin [*Coffea arabica*]	3,00E-16	3792,55
NODE_3529	gb|GQ435770.1|	*Caesalpinia sappan* Ycf5gene, chloroplast	1E-120	3742,42
NODE_8667	AEB33716.1	Conglutin beta 5 [*Lupinus angustifolius*]	2e-18	3706,49
NODE_2157	ref|XP_003610290.1|	Eukaryotic initiation factor iso-4F subunit p82-34 [*Medicago truncatula*]	1e-57	3661,68

Table 5. Continued

Contig	Accession	Description (BLASTX)	E-value	FPKM
NODE_9371	CU223764.1	Populus EST from leave	2,00E-21	3639,79
NODE_2450	ref|XP_002269959.2|	PREDICTED: peptidylprolyl isomerase domain and WD repeat-containing protein 1 [*Vitis vinifera*]	7,00E-51	3624,92
NODE_752	gb|EU428748.1|	*Glycine max* phytochrome A-3 (phyA) mRNA	3E-61	3603,74
NODE_28630	emb|X58710.1|	*M. sativa* mRNA for heat shock protein	3E-60	3412,64
NODE_920	gb|EU436322.1|	*Cogniauxia trilobata* rpl20-rps12 intergenic spacer, chloroplast.	5E-53	3399,38
NODE_3732	dbj|AB049336.1|	***Nicotiana tabacum* mRNA for dehydrin**	2E-14	3336,21
NODE_5029	unknown	unknown	unknown	3325,87
NODE_313	gi|356562555|	PREDICTED: eukaryotic initiation factor iso-4F subunit p82-34-like isoform 1 [*Glycine max*]	6,39E-25	3185,31
NODE_1241	gi|357513109|	Pre-mRNA splicing factor [*Medicago truncatula*]	9,26E-20	3163,18
NODE_28884	emb|CU223764.1|	Populus EST from leave.	3E-34	3127,44

It was carried out Gene Ontology annotation for all contigs found in both libraries (unprimed and osmoprimed seeds). Firstly they were categorized in 3 different functional groups: Biological Process, Cellular Component and Molecular Function. Secondly, they were subdivided in categories onto each group. Thus, it was determined the percentage of contigs in relation to the total of transcripts of each library.

It was observed 3155 contigs involved in Biological processes coming from the osmoprimed seeds library and 1116 contigs coming from the unprimed seeds library. In the osmoprimed library, 26% of contigs was related to Cellular Process, 25% to Metabolic Process and 10% to Biological Regulation. In the unprimed library it was verified the respective percentages of contigs in the same categories: 29%, 29% and 10%. Contigs distributed in biological adhesion, cell proliferation, locomotion and death were only found in osmoprimed seeds library (Figure 7).

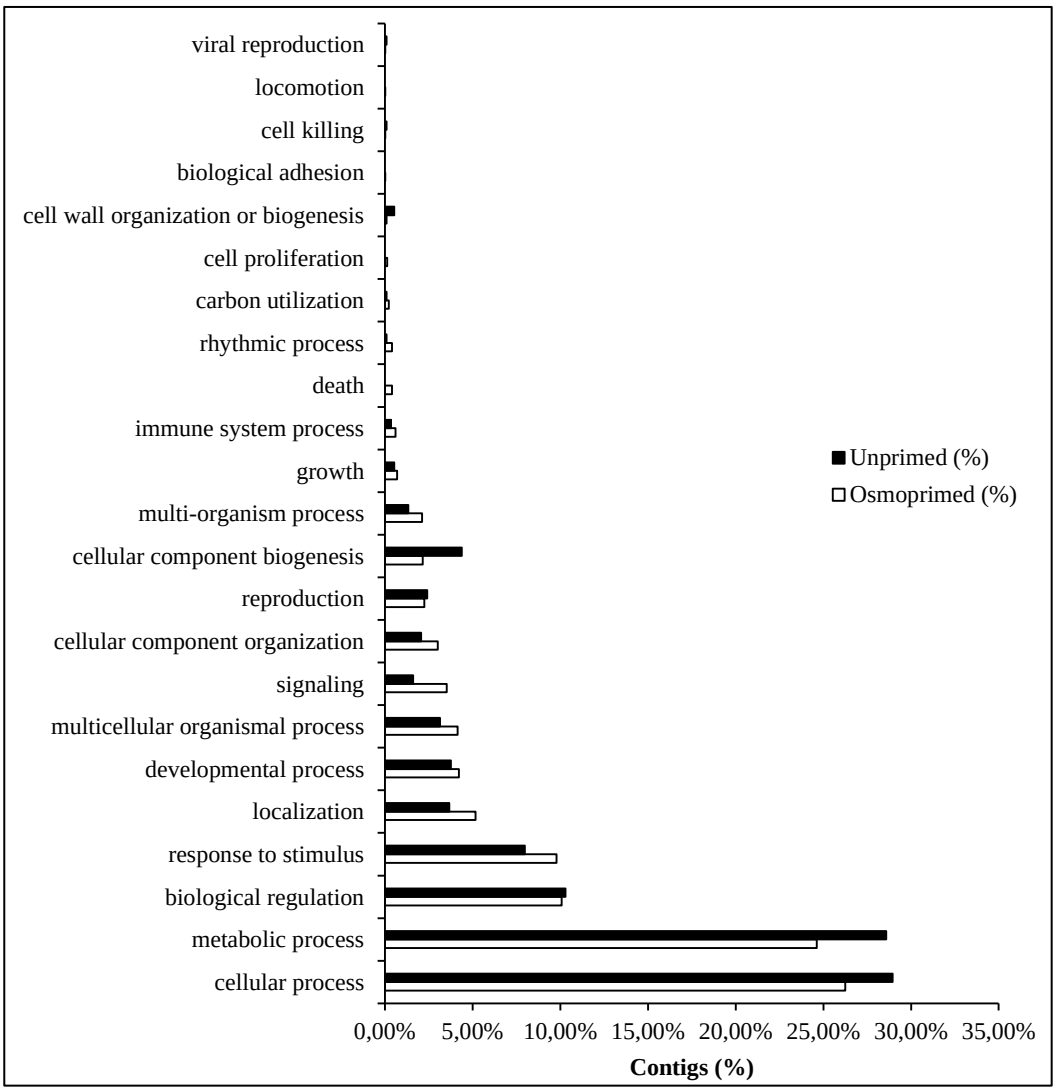

Figure 7. Division of contigs from both libraries (unprimed and osmoprimed seeds) in different Gene Ontology (GO) categories for Biological Processes.

It was found 2146 contigs in the osmoprimed seeds library related to the functional group Cellular Component, while in the unprimed seeds library it was observed 888. In the osmoprimed seeds library, 46% of contigs was grouped in cells, 35% in organelles and 13% in macromolecular complex. In the unprimed seeds library 43% of contigs was related to cells, 35% to organelles and 15% to macromolecular complex. Contigs distributed in symplast group, were only found in the unprimed seeds library (Figure 8).

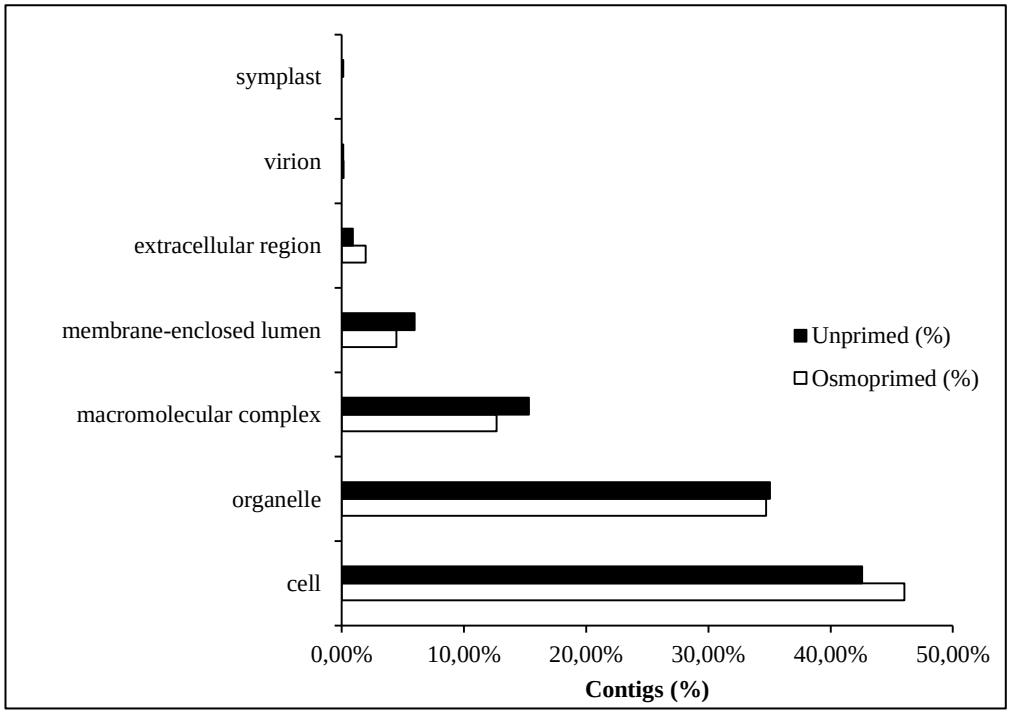

Figure 8. Division of contigs from both libraries (unprimed and osmoprimed seeds) in different Gene Ontology (GO) groups for Cellular Components.

It was found 1895 contigs in the osmoprimed seeds library with Molecular function, been 45% of them related to binding function, 35% associated with catalytic activity and 5% with transcription regulator activity. 641 contigs were found in the unprimed seeds library, been 43% correspondent to binding function, 38% to catalytic activity and 3% to transcription regulator activity (Figure 9).

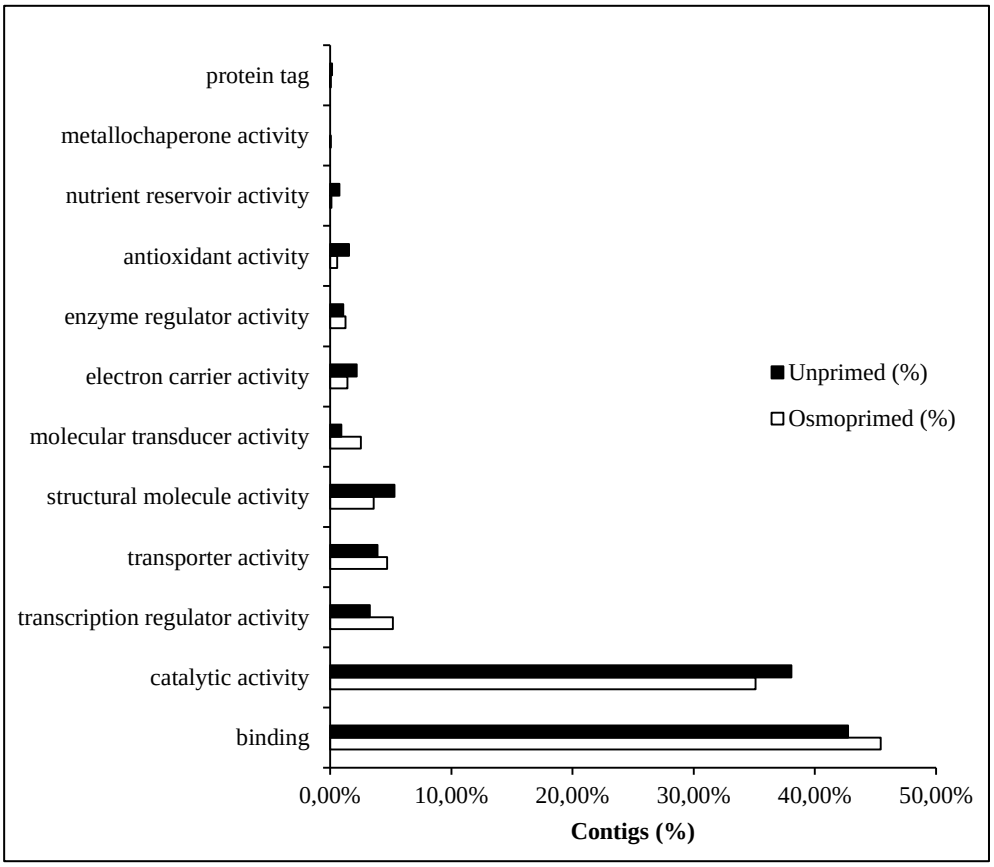

Figure 9. Division of contigs from both libraries (unprimed and osmoprimed seeds) in different Gene Ontology (GO) groups for Molecular Functions.

Gene expression assay by qRT-PCR

Total RNA samples used to generate cDNA (the starting material for qRT-PCR assays) presented high quality and high concentration (Table 6 and Figure 10).

Table 6. Concentration and quality (ratio $A_{260/280}$) of total RNA samples coming from unprimed and osmoprimed *P. pyramidalis* seeds.

Sample/Replicate	Concentration (ng/µl)	$A_{260/280}$
U2	227.36	1.82
U4	402.04	1.86
U5	319.08	1.85
O1	218.70	1.75
O3	301.82	1.71
O4	160.81	1.74

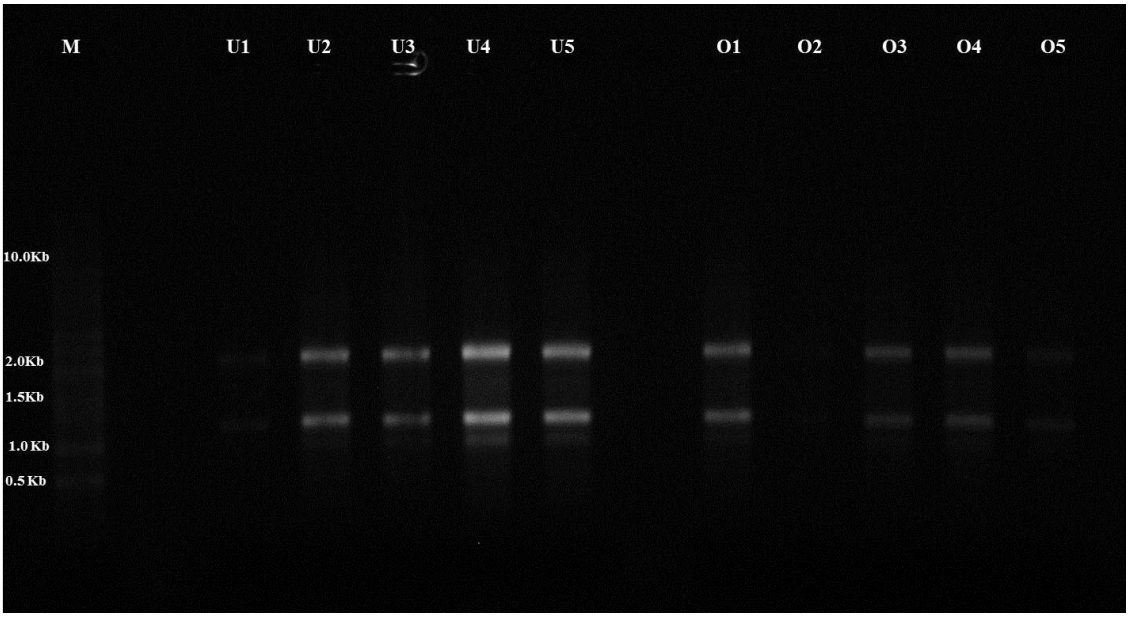

Figure 10. Total RNA samples isolated from osmoprimed and unprimed seeds and treated with DNAse enzyme, in 1.0% agarose gel. U1 to U5 = Unprimed samples. O1 to O5 = Osmoprimed samples. To downstream applications, it was just used three biological replicates of each treatment: U2, U4, U5, O1, O3 and O4.

To validate sequencing data revealed by RNA-seq, it was chose eight genes related to water stress (and subsequently with osmopriming) with recognized differential abundance in each library, comparing the relative expression of them in osmoprimed seeds (target sample) against unprimed seeds (calibrator), by qRT-PCR. Among them, six genes confirmed the tendency pointed out by RNA-seq data. The two genes used as reference (*Glycine max* serine/threonine protein phosphatase 2A (NODE 580) and Small GTP binding protein SAR 1 A (SAR 2) *Arabidopsis thaliana* (NODE 45602) to normalize the expression of the target ones, did not show significant differences in expression level by qRT-PCR and also showed high stability in the analysis proceeded in GeNorm software (M<0.05) (Table 7). The PCR products of all primers showed unique peaks in the melting curves (Figure 1, Appendix).

Table 7. Gene stability according GeNorm software.

Gene	Stability (M)	CV%
NODE 580	0.399	0.154
NODE 45602	0.387	0.140

*M<0.05

According RNA-seq data, **Seed maturation protein mRNA** (NODE 1142) was found with higher abundance in unprimed seeds library (FPKM = 3936) than in osmoprimed seeds library (FPKM = 3), but after qRT-PCR analysis it was demonstrated no significant differences in expression between osmoprimed and unprimed seeds (Figure 11 and Table 1).

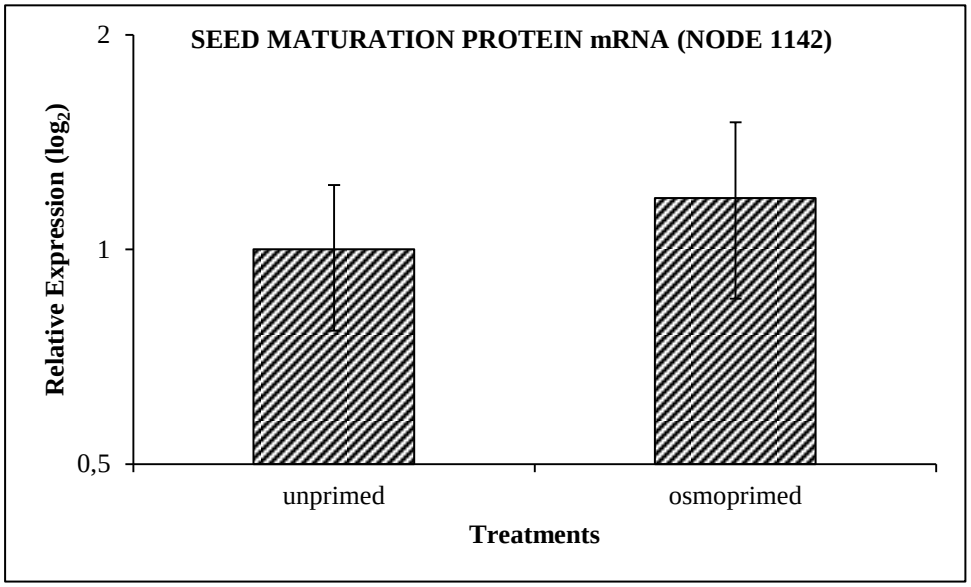

Figure 11. Relative expression of Seed maturation protein mRNA in osmoprimed seeds, determined by qRT-PCR Test t ($p<0.05$).

Oxidoreductase putative mRNA (NODE 46022) presented higher abundance in osmoprimed seeds library (FPKM = 384) than in unprimed seeds library (FPKM = 36) based on sequencing data. qRT-PCR assay revealed no significant differences in expression between osmoprimed and unprimed seeds (Figure 12 and Table 1).

Metallothionein-II protein mRNA (NODE 1074) showed higher abundance in unprimed seeds library (FPKM = 1102) than in osmoprimed seeds library (FPKM = 11) according RNA-seq data. qRT-PCR analysis confirmed these data, with higher expression level in unprimed seeds than in osmoprimed ones (Figure 13 and Table 1).

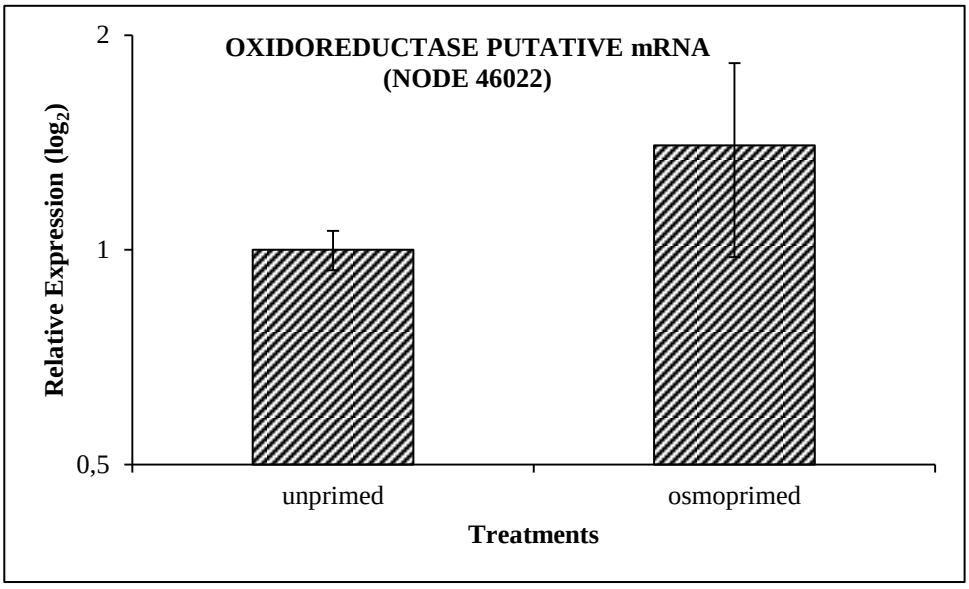

Figure 12. Relative expression of Oxidoreductase putative mRNA in osmoprimed seeds, determined by qRT-PCR. Test t ($p<0.05$).

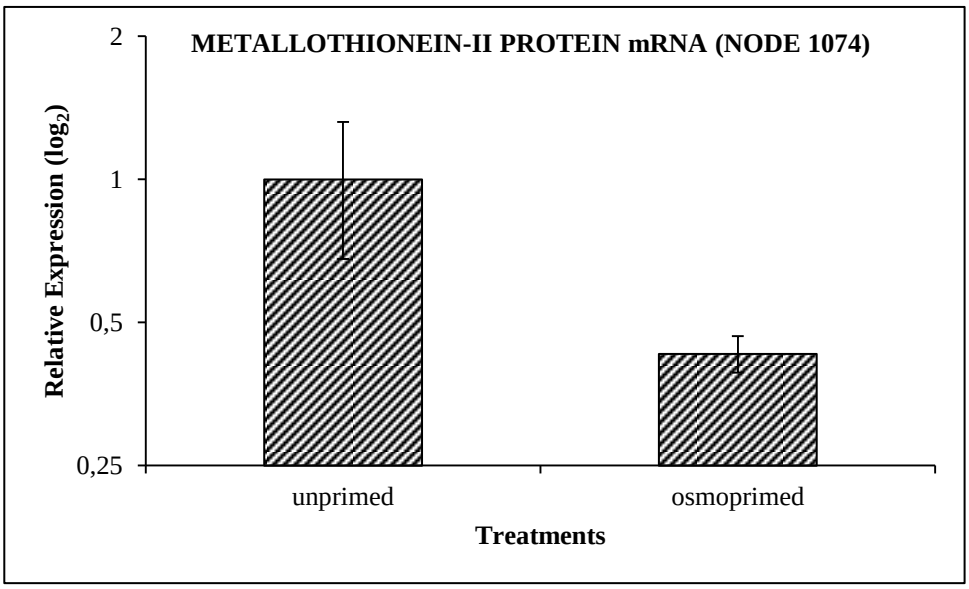

Figure 13. Relative expression of Metallothionein-II protein mRNA in osmoprimed seeds, determined by qRT-PCR. Test t ($p<0.05$).

Ca^{+2}-binding EF hand protein mRNA (NODE 86) was found with higher abundance in unprimed seeds library (FPKM = 2369) than in osmoprimed one (FPKM = 907). qRT-PCR experiment showed that there was no difference in expression level among unprimed and osmoprimed seeds (Figure 14 and Table 1).

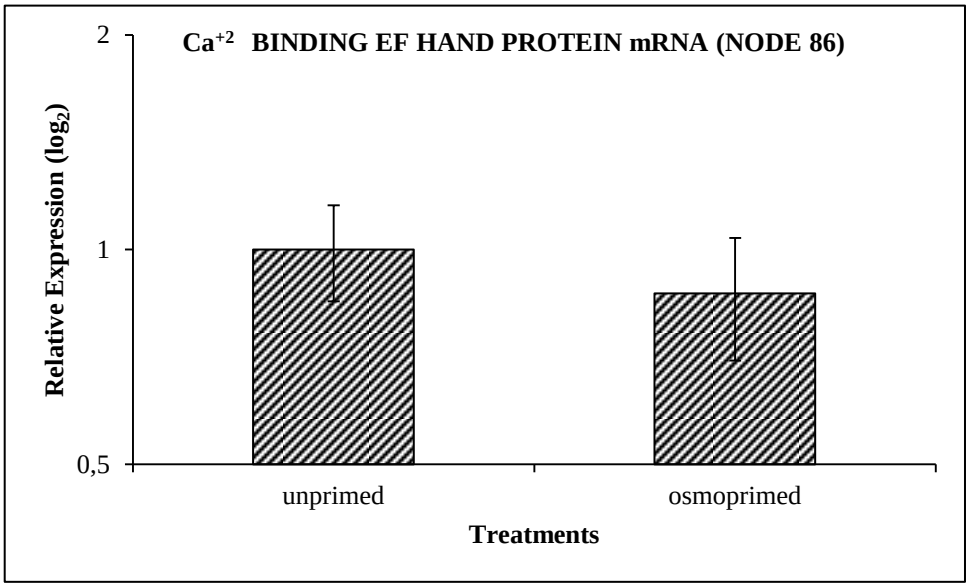

Figure 14. Relative expression of Ca^{+2} - binding EF hand protein mRNA in osmoprimed seeds, determined by qRT-PCR. Test t ($p<0.05$).

It was found more **Glutathione S-Transferase Tau 25** (NODE 37111) contigs in osmoprimed seeds library (FPKM = 1918) than in unprimed seeds library (FPKM = 17). qRT-PCR analysis confirmed higher expression level of this gene in osmoprimed seeds (Figure 15 and Table 1).

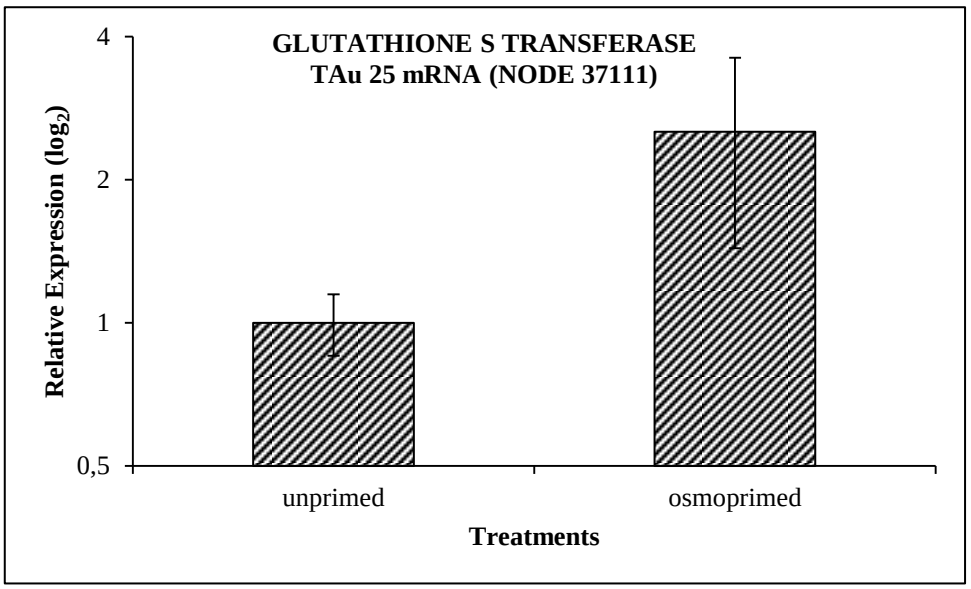

Figure 15. Relative expression of Glutathione S transferase Tau 25 mRNA in osmoprimed seeds, determined by qRT-PCR. Test t ($p<0.05$).

Auxin-induced glutathione S transferase mRNA (NODE 4738) had higher abundance in the osmoprimed seeds library (FPKM = 2843) than in the unprimed one (FPKM = 915) based on RNA-seq data, but the analysis of expression level via qRT-PCR indicated the other way around, with significant higher expression in unprimed seeds (Figure 16 and Table 1).

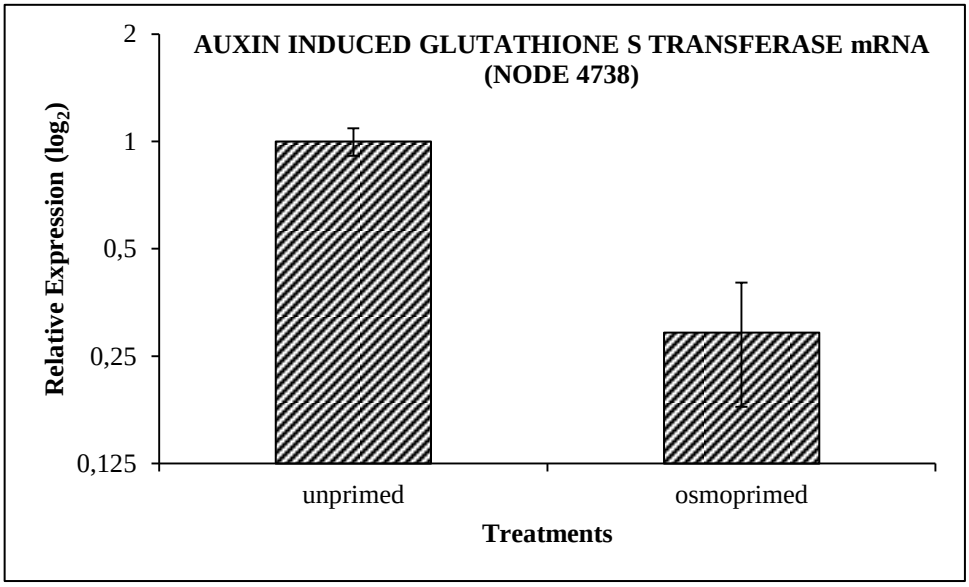

Figure 16. Relative expression of Auxin-induced glutathione S transferase mRNA in osmoprimed seeds, determined by qRT-PCR. Test t ($p<0.05$).

According sequencing data, **Tonoplast intrinsic protein alpha TIP** (NODE 753) contigs were exclusively found in the unprimed seeds library (FPKM = 4385) after subtraction, and qRT-PCR analysis showed higher expression of this gene in unprimed seeds (Figure 17 and Table 1).

Seed maturation protein mRNA (NODE 3870) was also exclusively found in the unprimed seeds library after subtraction process (FPKM = 3840), and qRT-PCR analysis showed higher expression of this gene in unprimed seeds (Figure 18 and Table 1).

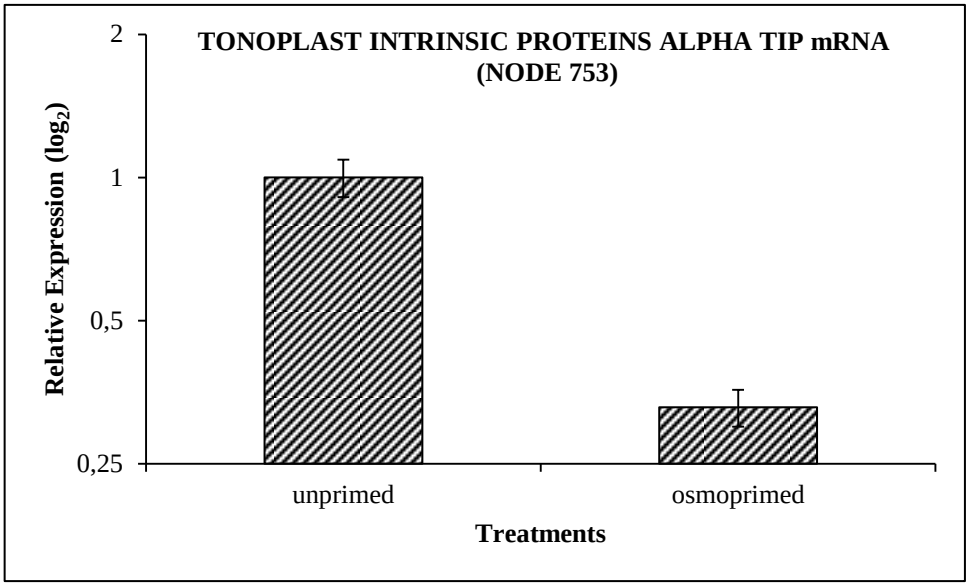

Figure 17. Relative expression of Tonoplast intrinsic protein alpha TIP mRNA in osmoprimed seeds, determined by qRT-PCR. Test t ($p<0.05$).

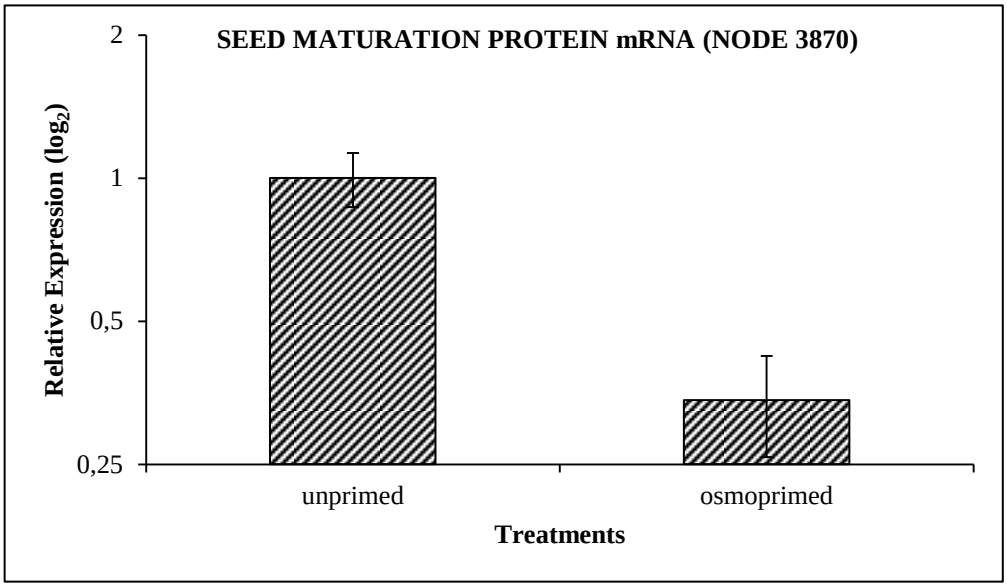

Figure 18. Relative expression of Seed maturation protein mRNA (NODE 3870) in osmoprimed seeds, determined by qRT-PCR. Test t ($p<0.05$).

DISCUSSION

Most abundant contigs in the subtraction libraries

It is known that seed priming is a pre-sowing partial hydration process that often improve crop performance (CHEN et al., 2012). Once an osmotic agent (as PEG) is used to control water uptake, seeds can experiment a kind of water deficit situation, which can alters gene expression and leads to especific gene induction, producing an increase of their transcripts and thus an increase of corresponding proteins (ROMO et al., 2001). Some authors have successfully identified differentially expressed genes in plant species under water stress or osmopriming conditions, combining SSH and Northern Blot analysis or SSH and microarray: Clement et al. (2008) (soybean), Zhang et al. (2007) (*Setaria italica*), Way et al. (2005) (wheat), Rodrigues et al. (2012) (soybean) and Cortez-Baheza et al. (2007) (*Capsicum annuum*). In this way, our proposal is innovative, since we used RNA-seq to identify differential expressed genes associated with osmopriming, followed by qRT-PCR to validate those data.

Among the thirty most abundant genes found in the especific osmoprimed seeds library, some had interesting relation to metabolic functions, what it is expected, since many germination-related events (gene transcription and translation, respiration and energy metabolism, early reserve mobilization, DNA repair) could occur during priming, contributing to improve rate, uniformity and final germination (CHEN et al., 2012; VARIER et al, 2010). At the same time those genes may offer tolerance to water deficit through activation of especific proteins and enzymes.

NODE 44560 showed high homology with alcohol dehydrogenase mRNA and was the second most abundant gene in the osmoprimed seeds library. Alcohol dehydrogenase (ADH) is a dimeric enzyme of the glycolytic pathway, found in mammals, yeasts and plants. The ADH found in plants requires zinc as a cofactor. The highest specificity of ADH in plants is for ethanol, aldehyde, and acetaldehyde substrates, but they can also utilize other primary alcohols as well. Plant ADH transcription has been demonstrated to increase by environmental stresses, such as dehydration (THOMPSON et al., 2010). A probably explanation for the induction of ADH mRNA in osmoprimed seeds library, is a reduced diffusibility of oxygen in the PEG solution, which could lead catingueira cells to invest in any strategy to get energy, in a low oxygen atmosphere like alcoholic fermentation. ADH is a key enzyme in this kind of fermentative metabolism, interconverting acetaldehyde in ethanol and producing

two molecules of ATP (TAIZ & ZEIGER, 2010). Zheng et al. (2004) studying differential expressed genes in water stressed (PEG) roots and leaves from maize, found increased expression of alcohol dehydrogenase transcripts after macroarray analyses. Zhang et al. (2007) evaluating some upregulated genes in *Setaria italica* roots submitted to dehydration stress, found induced Alcohol dehydrogenase I transcripts.

NODE 45006 described as a NAC domain protein (NAC27) mRNA had also higher expression in osmoprimed seeds library. NAC (NAM, ATAF1, ATAF2 and CUC2) domain comprises plant specific transcriptional factors that are involved in a variety of developmental events as well as in biotic and abiotic stress responses (as water stress). In soybean genome, NAC transcriptional factors are represented by at least 101 sequences (FARIA et al., 2011; NURUZZAMAN et al., 2010; PINHEIRO et al., 2009). In catingueira seeds the NAC mRNA is probably controlling the activation of responsive genes to osmopriming. Some authors found similar results in situations involving water stress: Faria et al. (2011) evaluating gene expression of *G. max* leaves under osmotic stress (PEG) (by qRT-PCR), found induction of NAC6 mRNA, which it is also associated with programmed cell death; Clement et al. (2008) observed that NAC2 protein gene was induced by drought in soybean nodules, making use of Northern Blotting analyses. Le et al. (2011) found a significant number of soybean GmNAC genes in response to dehydration in either roots or shoots or both tissues. Sang-Choon et al. (2008) in their microarray analyses also verified NAC transcription factors up-regulated in *Brassica rapa* plants submitted to drought.

NODE 43966 annotated as p-hydroxyphenylpyruvate dioxygenase mRNA was highly abundant in osmoprimed seeds library and is correlated to metabolism of tyrosine. Hydroxyphenylpyruvate acts as a precursor of homogentisic acid, which is involved in the biosynthesis of tocopherols and plastoquinones, and hydroxyphenylpyruvate dioxygenase (HPPD) is a key-enzyme on this process (FALK et al., 2002; FALK et al., 2003). Probably its expression in osmoprimed seeds can be a strategy to prevent an oxidative stress resulted from the initial water deficit. Soeda et al. (2005) through microarray analyses also found dioxygenase transcripts induced in osmoprimed *Brassica oleracea* seeds. Zheng et al. (2004) by macroarray analyses, observed increased expression of 4-hydroxyphenylpyruvate dioxygenase mRNA in leaves and roots of maize, as longer was the period of water deficit stress (PEG). Xue et al. (2011) analyzing the effect of *TaNAC69* overexpression in water stressed wheat seedlings observed that there was an increase of 4-hydroxyphenylpyruvate dioxygenase,

probably as a strategy to improve anti-oxidant activity. Soren et al. (2010) found some responsive genes to different abiotic stresses (as drought) in rice, through an EST library, and the transcript 4- hydroxyphenylpyruvate dioxygenase was one of these genes. Liu et al. (2010) produced two subtraction libraries from drought-stressed plants coming from tolerant and susceptible lines of *Lolium perenne* and observed in the reverse library (susceptible one), induced transcripts of 4- hydroxyphenylpyruvate dioxygenase.

NODE 44626 described as a double-stranded DNA-binding protein mRNA was also induced by osmopriming and could be related to nucleotide metabolism. DNA binding protein genes can encode transcription factors that can control expression (activation/repression) of target genes. In catingueira transcriptome this mRNA could be controlling the expression of osmopriming induced genes together with the NAC domain gene (other transcription factor). Under water stress, Zheng et al. (2004) observed that roots and leaves of maize maximized the expression of nucleic acid binding protein transcripts. Soren et al. (2010) also found a DNA binding protein gene in rice plants in response to drought, ABA, cold and high salinity.

Among the thirty most abundant genes found in the especific unprimed seeds library we can point out firstly that one related to Aquaporins group: NODE 753 (tonoplast intrinsic protein alpha TIP mRNA). Aquaporins are membrane – located water channels that control cell-to-cell water movement as well as plant cell expansion and organ development (CHEN et al., 2012). They have been isolated from tonoplast vacuolar membrane (Tonoplast Intrinsic Protein - TIP) and from the plasma membrane (Plasma Membrane Intrinsic Proteins - PIP) in different plant species (GAO et al., 1999). Among *Arabidopsis* TIPs orthologues, α-*TIP* is seed specific and seems to play a role in maintaining the integrity of tonoplast during dehydration process of seed maturation (GAO et al., 1999; MAUREL et al., 1997). Probably the TIP transcript found in this library, can be related with the expression of TIP aquaporins proteins in catingueira seeds during the dry stage, keeping up some basic vacuolar functions. High expression levels of a restricted number of tonoplast intrinsic protein (TIP) isoforms (TIP3;1 and/or TIP3;2, and TIP5;1) were identified by macroarray in *Arabidopsis* dry seeds (WILLIGEN et al., 2006). Li et al. (2008) by qRT-PCR and Gattolin et al. (2011) by fluorescent protein tagging and confocal microscopy found TIP3 transcripts in rice and *Arabidopsis* dry seeds.

There were three transcripts with exclusive occurrence in unprimed seeds library, related to LEA family: NODE 1471 annotated as desiccation protectant protein LEA 14 mRNA, NODE 3731 described as NtERD10A mRNA for dehydrin and NODE 3870 annotated as a seed maturation protein PM27. LEAs (Late embryogenesis abundant) are proteins produced in maturing seeds and their expression correlate with desiccation tolerance. Many LEAs are also induced in response to abiotic stress condition in seeds. They protect cellular structures from the effects of water loss by sequestering ions, by direct protecting other proteins, by renaturing unfolded proteins, by protecting membranes or acting as a hydration buffer (BATTAGLIA et al., 2008; WISE & TUNNACLIFFE, 2004). NODE 1471 is part of LEA group 4 and encodes LEA proteins with special properties: capacity to bind to water molecules, stabilize membrane surface and function as a salvation film (SOREN et al., 2010). NODE 3731 is found in LEA group 2, and encodes proteins that accumulate during seed desiccation and in response to water deficit, low temperature or salinity (BATAGGLIA et al., 2008). Hundertmark et al. (2011) found the highest dehydrin transcripts abundance in *A. thaliana* dry mature seeds by qRT-PCR assays. Shih et al. (2010) by hybridization *in situ* analyses verified increased expression of 7 hydrophilic LEAs transcripts in different seed tissues of soybean, after natural process of drying. Haider et al. (2012) found two dehydrin mRNA transcripts during the late stage of development of pea seeds, associating them to desiccation tolerance.

NODE 3870 was also exclusively found in unprimed seeds library. This kind of transcript is really typical in mature dry seeds, since they accumulate during the final stage of seed development (maturation), to guarantee tolerance to the subsequent desiccation phase. According Batagglia et al. (2008) those transcripts are included in LEA group 5, which comprises LEA proteins that contain a significantly higher proportion of hydrophobic residues. Beilinson et al. (2005) produced two subtraction libraries using as starting material soybean seeds 7 and 21 days after flowering. They found seed maturation protein transcripts, with differential expression, in soybean seeds collected 21 days after flowering. Soeda et al. (2005) observed induction of seed maturation protein transcripts in fully mature *Brassica oleraceae* seeds, classifying them in the LEA superfamily. A seed maturation protein gene was highly expressed in mesophyll tissues of soybean seeds 35 days after flowering (GmPM5), which means accumulation of this mRNA during late embryogenesis (SHIH et al., 2010).

Gene expression

Among the eight target genes chosen to study gene expression and thus validate SSH results, some were exclusively found in the osmoprimed seeds library, some others exclusively found in the unprimed seeds library and other ones found in both libraries.

NODES 1142 and 3870 presented high homology with a seed maturation protein mRNA. These transcripts are part of LEA family (group 5) that encodes insoluble proteins (with globular conformation) that accumulate at the end of seed development, protecting cell membranes and other proteins during desiccation phase (BATAGGLIA et al., 2008). We already mentioned some works with increased expression of seed maturation protein transcripts in dry seeds (SHIH et al., 2010; BEILINSON et al., 2005; SOEDA et al., 2005). The relative expression of the first transcript (NODE 1142) did not confirm SSH results (higher abundance in unprimed seeds library), since we could not observe the expected higher expression of it in unprimed seeds. The second transcript (NODE 3870) was exclusively found in unprimed seeds library and qRT-PCR assay showed significant higher expression in unprimed seeds. This can be explained because qRT-PCR technique does not consider the subtraction effect, thus we can not find genes with exclusive expression in any of the two treatments proposed.

NODE 46022, an Oxidoreductase putative gene with higher expression in osmoprimed seeds, is related to the expression of Oxidoreductase enzymes. Generally, the enzymes that transfer electron from one molecule to another are called Oxidoreductase. Because most of metabolic oxidation reactions involve removing of hydrogen from the electron donor, these enzymes are called dehydrogenases. The term oxidase is used only for the enzymes in which the oxidation reaction involves molecular oxygen (O_2) participating as electron acceptor. The expression of this transcript confirms that catingueira metabolism is reinitiated in osmopriming condition. This could explain why osmoprimed catingueira seeds germinate faster. Authors like, Nakashima et al. (2007) found induction of a NAD(P)H-dependent Oxidoreductase in transgenic rice plants over-expressing OsNAC6 under water stress condition. Porth et al. (2005) produced two subtraction libraries from cell suspension cultures of *Quercus petraea* callus, under 1h or 2 days of hyperosmotic stress condition, and identified among the ESTs an Oxidoreductase, 2OG-Fe(II) oxygenase family.

NODE 1074 described as a Metallothionein-II protein mRNA is part of Metallothioneins (MTs) group. MTs are low-molecular weight cysteine (Cys)-rich proteins that can effectively bind metals via their Cys residues. Based on the

arrangement of Cys residues, MTs have been divided into three classes: Class I that includes only MTs from mammalians and vertebrates; Class II that contains all other MTs from plants, fungi, and invertebrate animals and Class III that is enzymatically derived polypeptides with a poly (gamma-Glu-Cys)-glycine structure (ZHOU et al., 2012). Many genes encoding MTs have been isolated and characterized in plants, but the precise functions of MTs remain to be established. Evidences suggest that plant MTs play a role in maintaining the homeostasis of essential metal ions, detoxifying heavy metals, scavenging reactive oxygen species (ROS) and repairing plasma membrane (XIA et al., 2012; HALL et al., 2002). In plants, MTs are also involved in drought response (PEG 6000) (YANG et al., 2009) and seed development (YUAN et al., 2008). The higher expression of them in unprimed seeds could be a strategy to prevent oxidative stress, preparing the cells to the further hydration phase. Beilinson et al. (2005) found in their subtracted library (21 days after flowering) some metallothionein transcripts, what means that at the end of the development, soybean seeds can accumulate this kind of gene, as in unprimed catingueira seeds. Zhou et al. (2012) analyzing EST libraries from sacred lotus found that NnMT2a comprised approximately 1.17% of the total transcripts in embryonic axes of late maturation seeds. Ren et al. (2012) through transcriptional analyses showed that two *Arabidopsis* metallothionein genes (*AtMT4a* and *AtMT4b*) are specifically expressed in late embryos.

NODE 86 (Ca^{+2}-binding EF hand protein mRNA) is part of the superfamily EF-hand calcium binding proteins that regulates diverse cellular activities ranging from exocytosis, metabolism, transcription, fertilization and cell proliferation, to Ca^{2+} buffering and homeostasis. EF-hand calcium sensors (such as calmodulin) translate the physiological changes in calcium levels into specific cellular responses by undergoing a large conformational change that exposes a binding site recognized by downstream effectors (BHATTACHARIA et al., 2004). They also have been shown to be involved in membrane trafficking and associated to microtubules organization (ANDRADE et al., 2004). The higher expression of this gene in unprimed seeds might be a result of accumulation during seed maturation, been possibly involved in early physiological responses after imbibition.

NODE 37111 was described as a Glutathione S Transferase TAU 25 (GTS). The GSTs compose a class of enzymes with potential antioxidant properties. They can act as antioxidants by tagging oxidative degradation products (especially from fatty acids and nucleic acids) for removal or by directly scavenging peroxides. GSTs are also well

known for the capacity to detoxify xenobiotics (such as herbicides), and respond to a range of biotic and abiotic stresses (as water deficit). They have been divided into six classes: tau, phi, zeta, theta, lambda, and DHAR (for glutathione-dependent dehydroascorbate reductase) (DALTON et al., 2009; GONG et al., 2005) and the identified GST transcript in osmoprimed catingueira seeds encodes enzymes found in class TAU. The antioxidant function of this enzyme, is fundamental to prevent oxidative stress in catingueira seeds under osmopriming condition, contributing to the increased vigour of them. Irsigler et al. (2007) identified by microarray, some Glutathione S transferase transcripts up-regulated in *Glycine max* plants submitted to osmotic stress treatment (PEG 8000). Soeda et al. (2005) found in *B. oleracea* seeds Glutathione transferase genes associated to osmopriming condition. Jia et al. (2006) identified by macroarray and scatter plot Glutathione S transferase mRNA in PEG-treated seedlings of maize.

NODE 4738 annotated as an Auxin-induced glutathione S transferase and found with higher abundance in unprimed seeds library is a gene whose expression is dependent of the plant hormone auxin. This hormone regulates growth and development processes (such as seed development) by stimulation of cell expansion, cell division, cell differentiation or by controlling the expression of auxin-responsive genes (SCHENCK et al., 2010; PARK et al., 2003), such as the Glutathione S transferase gene, which has direct relation with antioxidant function, especially in case of abiotic stress condition (DALTON et al., 2009). However, the relative expression of this gene showed that the expression of it was higher in unprimed seeds.

NODE 753 annotated as a Tonoplast intrinsic protein alpha TIP is classified into Aquaporins group and was already described according its function in water transport (CHEN et al., 2012). It was exclusively found in unprimed seeds library, and presented higher expression in unprimed seeds. Again this can be explained by the fact that qRT-PCR analysis does not consider the subtraction effect. Authors like Gattolin et al. (2011) and Li et al. (2008) also found higher expression of TIP genes in dry seeds.

CONCLUSIONS

Suppressive subtractive hybridization is a powerful technique to identify a large number of differentially expressed genes, revealing many gene sequences with high quality, but in case of this approach the tool chosen to validate SSH data (qRT-PCR),

showed that this technique has some limitations, since the relative expression of some genes did not confirmed SSH findings. We suggest that probably the subtraction libraries were not completely saturated.

It is necessary to select some new other genes to evaluate their relative expression level, especially related to the osmopriming situation (the target condition of this study) and during germination under stress.

The genes found in the osmoprimed seeds library that had their expression confirmed by qRT-PCR might explain how catingueira seeds improve their vigour under osmopriming condition.

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CONCLUSÕES GERAIS

A partir dos dados obtidos com o presente trabalho pode-se concluir que:

- *Poincianella pyramidalis* tem ampla plasticidade ecológica, germinando bem em diferentes temperaturas, o que condiz com sua larga distribuição geográfica ao longo do Nordeste Brasileiro.

- A espécie apresenta moderada tolerância ao estresse osmótico, mantendo a germinação de suas sementes até o potencial osmótico -1.0 MPa e formação de plântulas normais até o potencial osmótico -0,6 MPa.

- O osmocondicionamento promove melhorias na germinação de sementes de *Poincianella pyramidalis*, não compromete a longevidade de sementes armazenadas (em condições ideais) e é capaz de reverter os efeitos negativos do envelhecimento acelerado a 40ºC.

- O osmocondicionamento não é capaz de reverter o efeito negativo de condições extremamente estressantes (elevadas temperaturas e potenciais osmóticos consideravelmente negativos) sob a germinação das sementes de *Poincianella pyramidalis.* O desempenho das mudas de catingueira oriundas de sementes osmocondicionadas, também não é maximizado pelo uso do osmocondicionamento, provavelmente pelo fato do lote utilizado ser recém-coletado, e prescindir o uso da técnica.

- A utilização da técnica de SSH auxilia na identificação de genes que estão envolvidos no aumento de vigor de sementes de *Poincianella pyramidalis*, porém a técnica apresenta algumas limitações, as quais foram comprovadas através da validação via qRT-PCR, que aponta alguns genes que não foram completamente subtraídos.

- A seleção de novos genes com expressão diferencial induzida pelo osmocondicionamento faz-se necessária, para uma melhor avaliação das bibliotecas obtidas através da hibridização supressiva subtrativa.

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RESUMO GERAL:

A formação de sementes de alta qualidade é pré-requisito básico para geração de plântulas vigorosas, que tolerem os diversos tipos de estresses bióticos e abióticos (como seca e calor) que limitam o rendimento e a produtividade agrícolas. Neste contexto, o uso de técnicas que possam aumentar o vigor de sementes (como o osmocondicionamento) pode ter um efeito positivo na produção vegetal. Este trabalho objetivou estabelecer o comportamento germinativo de sementes de *Poincianella pyramidalis* (catingueira) em diferentes condições de temperatura e potenciais osmóticos, assim como determinar as bases moleculares e fisiológicas envolvidas na resposta das sementes ao osmocondicionamento. Os ensaios envolvendo incubação das sementes de catingueira em diferentes temperaturas (20, 25, 30, 35, 37 e 40ºC) e potenciais osmóticos (0,0; -0,2; -0,4; -0,6; -0,8; -1,0; -1,2 MPa) mostraram que a espécie apresenta uma ampla plasticidade ecológica (visto que a mesma é capaz de germinar em todas as temperaturas estudadas) e moderada tolerância ao estresse osmótico (a germinação foi mantida até o potencial de -1.0 MPa e a formação de plântulas normais até o potencial de -0,6 MPa). O osmocondicionamento aumenta o vigor de sementes de catingueira (velocidade e uniformidade de germinação são potencializadas), reverte os efeitos negativos do envelhecimento acelerado a 40ºC (porém não a 45ºC), não compromete a longevidade de sementes armazenadas por dois anos em geladeira, não é capaz de reverter os efeitos de condições extremamente estressantes (como potenciais demasiadamente negativos e temperaturas elevadas) e não influencia na emergência e desenvolvimento de plântulas de catingueira. Vários genes estão envolvidos no aumento do vigor de sementes de catingueira após aplicação do osmocondicionamento. Esses genes estão relacionados a funções metabólicas que são reativadas na condição do osmocondicionamento. As informações geradas a partir desta proposta servem de base para o estudo de outras espécies do Semi-árido Nordestino Brasileiro.

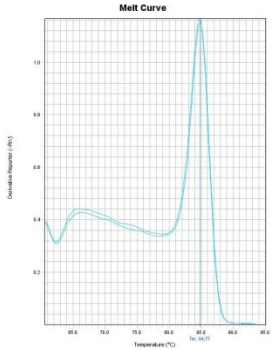
Palavras-chave: Catingueira, temperatura, estresse osmótico, osmocondicionamento, expressão gênica.

GENERAL ABSTRACT:

The formation of high quality seeds is a basic pre-requisite to generate vigorous seedlings, that tolerate the various types of biotic and abiotic stresses (as drought and heat) which limit agricultural yield and productivity. In this context, the use of techniques that increase seed vigour (as osmopriming) might have a positive effect on plant production. This approach aimed to establish the germinative behavior of *Poincianella pyramidalis* (catingueira) seeds in different conditions of temperature and osmotic potentials, as well as to determine the molecular and physiological base involved in the response of the seeds to osmopriming. The assays involving incubation of catingueira seeds in different temperatures (20, 25, 30, 35, 37 e 40ºC) and osmotic potentials (0,0; -0,2; -0,4; -0,6; -0,8; -1,0; -1,2 MPa) showed that the species has a wide ecology plasticity (once it is able to germinate in all studied temperatures) and moderate tolerance to osmotic stress (germination was kept until -1.0 MPa potential and formation of normal seedlings until -0.6 MPa potential). Osmopriming increases catingueira seed vigour (rate and uniformity of germination are maximized), reverts the negative effects of accelerated ageing at 40 ºC (but not at 45 ºC), does not compromise longevity of seeds stored for two years in the fridge, does not revert the effects of extremely stressful conditions (as overly negative osmotic potentials and higher temperatures) and does not influence in emergence and development of catingueira seedlings. Many genes are involved in the improvement of catingueira seed vigour after osmopriming application. These genes are related to metabolic functions that are reactivated in the osmopriming condition. The information generated from this proposal serves as a base to study others species from the Brazilian Northeastern Semi-arid.

Key-words: Catingueira, temperature, osmotic stress, osmopriming, gene expression.

APÊNDICE



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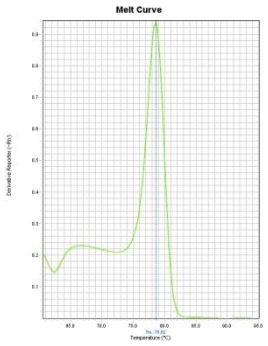

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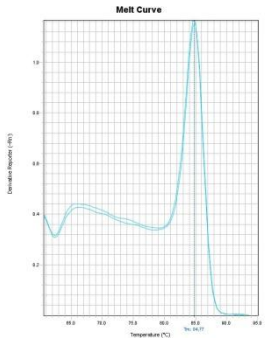

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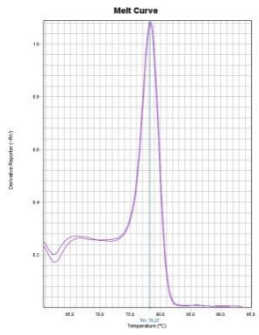

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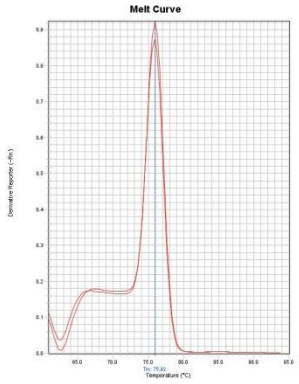

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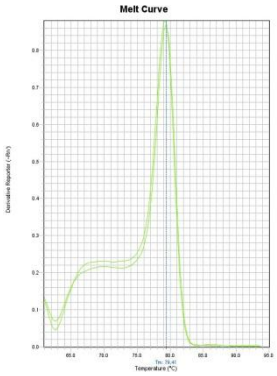

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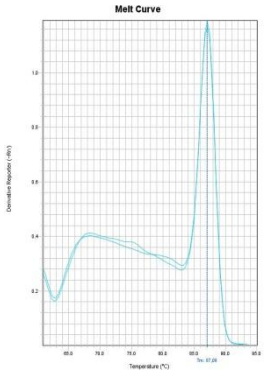

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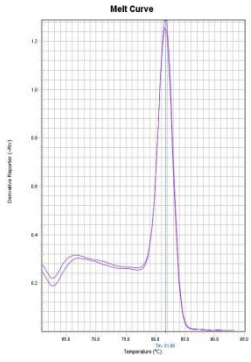

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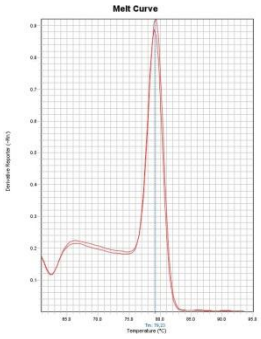

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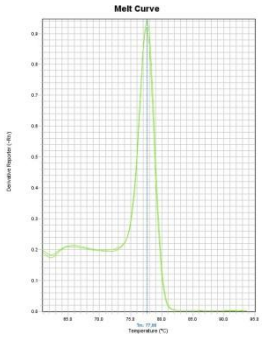

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Figure 1. Melting curves of amplicons coming from different primes.