Induction of Desiccation tolerance in *Araucaria angustifolia* seeds.

M.Sc. Thesis Report

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Msc thesis plant physiology
Course code**: 80436**

May, 2015

Master thesis research submitted as fulfilment of the requirement of the degree of Masters of Plant Sciences at Wageningen University

Table of Contents

List of figures ii

List of tables iii

ABSTRACT iv

INTRODUCTION 1

MATERIALS AND METHODS 4

Seeds 4

Dehydration method and moisture content determination 4

Antioxidant pre-treatments 4

Viability test 5

Extraction and derivatization of primary metabolites for GC-TOF-MS analysis 5

GC-MS data processing and compound identification 6

Statistical analysis 6

RESULTS 7

Moisture content 7

Viability test in response to antioxidants 7

Metabolome composition of *A. angustifolia* embryos 8

Metabolomic difference during dehydration/rehydration cycle due to antioxidant treatment 9

Response of metabolites to desiccation and antioxidants 10

DISCUSSION 12

Effect of antioxidant treatment in desiccated tissue 13

Overall changes of metabolites in response to desiccation and antioxidants 14

CONCLUSION AND FUTURE PERSPECTIVES 16

ACKNOWLEDGEMENT 17

REFERENCES 18

List of figures

Figure 1. Moisture contents of desiccated mega gametophyte of *A. angustifolia* seeds when dried at a different time points at 22C and 30% RH...7

Figure 2. Variation of viability of desiccated mega gametophyte and embryo of *A. angustifolia* in trolox and dried in different time points (0, 4 and 24 hours) at 22°C and 30% RH...8

Figure 3. Functional Categorization of 76 known and unknown metabolites detected in *A. angustifolia* embryo over three different states (hydrated, dehydrated and rehydrated)..................................9

Figure 4. Heatmap clustering representation of metabolite-antioxidant correlations in response to desiccation treatment in embryo samples of A.angustifolia..12

Figure 5. Hierarchical Heat map representation of the metabolite-metabolite correlations in response to trolox and desiccation treatments in embryo samples of A. *angustifolia*..................................14

List of tables

Table 1. Metabolites identified from a methanol tissue extract of Araucaria embryos.....................9

ABSTRACT

Antioxidant system is believed to function as a protector of viable seeds against oxidative damage; it regulates metabolic activities by counteracting the detrimental free radical of oxygen species produced during desiccation. To test the mechanism of antioxidant in acquisition of desiccation tolerance in seeds, we assessed the changes of metabolites of recalcitrant seeds during desiccation. We used the embryo of *Araucaria angustifolia* to study the effect of water soluble antioxidant (trolox) on metabolite changes in three different states (hydrated, dehydrated and rehydrated). Our findings suggest that treatment of embryo with trolox before drying may rescue the viability of seed. Furthermore heatmap clustering shows that the abundances of sugar metabolite was up regulated in hydrated embryo due to application of trolox and decrease during drying and rehydrated. On the other hand the abundances of amino and organic acids had a similar trend of increasing from hydrated to rehydrated embryo regardless of either treated with trolox or not. However, myo-inositol display different pattern compared to other sugar metabolites. Myo-inositol were highly accumulated in rehydrated embryo sample treated with trolox while was depleted in non-treated rehydrated embryo compared to dried embryo. This observation controversy with suggestions from other research studies thereby they found highly significant correlation between the levels of myo-inositol and accumulation of raffinose family oligosaccharides (RFOs). Also the desiccated embryo treated with trolox was not being able to grow therefore this controversy open a new questions for further research.

INTRODUCTION

Seeds of plant species vary in sensitivity to desiccation, which makes them to be categorized into two terms. Orthodox seeds cease metabolism during maturation stage so that can be dried to very low moisture content without irreversible damage (Stanislawa Pukacka, Malec, & Ratajczak, 2011) and recalcitrant seeds are characterized with high moisture contents and remain metabolically active even after shedding, thus they cannot withstand very low moisture contents during dehydration (Leprince, Buitink, & Hoekstra, 1999; Vertucci & Farrant, 1995). Despite the fact that orthodox seeds can tolerate low moisture content, but there are limits to seed drying below which all types of seeds lose their viability (Patricia Berjak, 2006; Walters, Hill, & Wheeler, 2005). Recalcitrant seeds are regarded as desiccation sensitivity seeds due to lack of mechanism of DT during seed maturation. These types of seeds are sensitive to dehydration during development and after maturation (i.e. when they are shed from the parent plant). Such type of seeds quickly lose their germination ability when are dried below a certain relative value of moisture contents depending on the species (Stanislawa Pukacka et al., 2011). Their inability to survive at very low moisture contents renders them unsuitable for medium to long term seed storage compared to orthodox seeds (Li & Pritchard, 2009). This limited storage of recalcitrant seed become a significant problem in the maintenance of genetic seed bank for long term conservation (Hendry et al., 1992). Imbalance between production of reactive oxygen species (ROS) and ability to scavenge them thought to be a source of viability loss in recalcitrant seeds during dehydration. The involvement of ROS in viability loss of desiccated seeds has been suggested by different researchers. Ability of orthodox seeds to withstand desiccation might be related to to the removal or counteraction of ROS produced by using antioxidant system present in plant cells (Bailly, 2004; Stanislawa Pukacka et al., 2011). Furthermore (Patricia Berjak & Pammenter, 2008) recommend fast drying of embryos to shorten the time of ROS-associated damage to accumulates thus prevent metabolism that induced damage. Even though the relationship between the viability loss of recalcitrant seeds and oxidative stress during desiccation has been investigated by several researchers (Greggains, Finch-Savage, Atherton, & Berjak, 2001; Stanisława Pukacka & Ratajczak, 2006) but the metabolic basis of desiccation sensitivity of recalcitrant seeds are still unknown, which pause a challenge to determine appropriate measures to better conserve these seeds.

Basically, the antioxidant defence categorized into three classes (a) Liposoluble vitamins i.e Lutein and α-tocopherol (b) water soluble reductants i.e glutathione, ascorbate and trolox (c) Enzymatic antioxidants i.e. superoxide dismutase (SOD), catalase and peroxidases. Among the three classes of antioxidant, only water soluble reductants seem to be effective mechanism in the detoxification process within the plant cells in which the hydrogen peroxide (H_2O_2) is scavenged (Francini et al.,

2006). However, the water soluble antioxidant appears to be vital in photosynthetic tissues but in recently comparatively few studies have been performed in seeds. According to (Navari-Izzo, Quartacci, & Sgherri, 2002) addition of tocopherol and lipoic acid in glutathione/ascorbate cycle might increase inhibition of oxidative damage to cell membranes. It prevents the membrane damage by trapping the lipid radicals and suppressing the lipid peroxidation rather than scavenging oxygen singlet. Apart from antioxidant defence, metabolites such as carbohydrates and its derivatives also suggested to promote acquisition of DT during dehydration/rehydration cycle (Bartels & Salamini, 2001; Hoekstra, Golovina, & Buitink, 2001). Primary metabolites like sugars, amino acids, nucleotide derivatives, lipids, polyamines and defense compounds hypothesized to protect the membrane of cell from damage during dehydration. The accumulation of sugar metabolites such as raffinose, stachyose, sucrose and low amount of monosaccharide in orthodox seeds are accompanying with the acquisition of DT during maturation drying. These sugar metabolites might facilitate formation of glassy state for thereby preventing phase transitions in the lipid bilayer (Crowe, Hoekstra, & Crowe, 1992). Though, for seeds to acquire full DT, multifactorial mechanisms inducing DT during maturation should be considered. It is possible that several regulatory pathways act in parallel and interact with one another to induce DT in seeds.

Antioxidants system as long as been associated with acquisition of DT like other mechanisms particularly metabolites changes, thus the ability of antioxidants to protect membrane tissue might interact with the changes of metabolites during dehydration/rehydration of seed. In recent studies the change of metabolites and capacity to scavenge ROS seems to be of particular interest, because during development stage of orthodox seeds ROS are formed from respiratory electron transport but they are scavenged by present antioxidant system (Leprince, Harren, Buitink, Alberda, & Hoekstra, 2000; Pammenter & Berjak, 1999). Moreover, reviews of many studies show that the production of ROS that induce membrane damage is linking with the decrease of some metabolites during dehydration hence ROS is considered as a major injurious factor in recalcitrant seeds. For instance (Francini et al., 2006) observed the higher levels of ROS accumulated in the embryo of *A. angustifolia* might be due to occurrence of many metabolic events, which results depletion of carbohydrates that constitute membrane protective metabolites such as sugar. A series of cascade reactions without mechanism to quench these free radicals (ROS) may be set in motion that can lead to an increase in lipid peroxidation, membrane damage and eventually cell death (Bailly, 2004; Hendry, 1993). These reactions have been connected with seed deterioration and viability loss in recalcitrant seeds during dehydration (Bailly, 2004; Pammenter & Berjak, 1999). Therefore, loss of viability of recalcitrant seeds during dehydration can be due to oxidative damage induced by ROS produced, mis-regulation of antioxidant systems curbing ROS and concomitantly changes of protective metabolites within the

tissue. Together, then, these factors must be seriously considered as constituting one of the major causes of viability loss in recalcitrant seeds.

In the present study, our goal was to study the protective role of antioxidants associated with metabolites changes in acquisition of DT during dehydration. In addition, we aimed to introduce the model of establishment of DT in *Araucaria angustifolia* as a valuable tool to rescue the recalcitrant seeds from viability lost. To conduct this study we used embryos from *Araucaria angustifolia seeds*, in which published to belong in recalcitrant category. *Araucaria angustifolia* seeds cannot be dehydrated below 15% of moisture content without loss of their germination capacity (Francini et al., 2006). Although the natural target of artificial dehydration process in recalcitrant seed is embryonic axes (Leprince et al., 1999), In *A. angustifolia* seeds mega gametophyte also plays an important role in protecting embryonic axes. In fact the embryonic axes are encloses with two long embryos which surrounded by mega gametophyte. Embryos and mega gametophyte supplies both mechanical protection and nutrients reserves during radicle germination. Since desiccation and consequent oxidative stress in seeds may compromise the mobilization of nutrient reserves from mega gametophyte/embryos thereby cut off nutrients supply and ensuing embryo death. Therefore in determination of physiological response to trolox and desiccation we used both isolated mega gametophyte and embryo. Therefore to better understand the metabolic basis change due to treatment of antioxidant and how play role in acquisition of DT, the study of metabolome of *A. angustifolia* was compared over three different states of moisture contents (hydrated, dehydrated and rehydrated).

MATERIALS AND METHODS

Seeds

In 2014, seeds of *Araucaria angustifolia* were collected from areas of natural occurrence of the species in the forest of the municipality of Aiuruoca, MG (21 ° 13'55,0"S coordinates; 44 ° 58'42,0" W), which classified as rain forest Low-Montana in Brazil (Oliveira-Filho & Fontes, 2000). The seed collection held between the months of April and May, by collecting seeds of at least 12 mother trees based on recommendations. Seeds sorting and processing was done at Minas Gerais/Brazil–Federal University of Lavras then a mild drying is conducted at room temperature (25 ± 2°C) to remove surface water from the seeds resulting from the processing. The structure of Araucaria seed is 3-8cm long, 1-2cm wide and is composed of thick seed coat surrounding a large mega gametophyte which encloses a straight embryonic axis with two long cotyledons. In this research study, we conducted experiment by using isolated mega gametophyte and embryo to avoid the possible effects of seed coat on the loss of viability during desiccation treatments.

Dehydration method and moisture content determination

Mega gametophyte disc (1cm diameter) and embryo were isolated from the seeds and dried in a closed humidity cabinet at 22°C and 30% RH in a monolayer on a mesh tray with air flow to induce rapid desiccation. To determine moisture contents, approximately of 9 discs from mega gametophyte and 9 embryo of *A. angustifolia* weighed and allowed to dehydrate in in different time points (4 and 24 hours) at 22°C and 30% RH then weighed again before subjected to oven (105°C) for 24 hours to determine dry weight. Moisture contents were calculated on a dry weight basis and dry weight obtained through oven drying embryoss and mega gametophyte at 105°C for about 24 hours. The results expressed as the percentage of water contents obtained from three replicates ± standard deviation (SD).

Antioxidant pre-treatments

Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid, $C_{14}H_{18}O_4$) is a water-soluble analog of vitamin E with molecular weight of 250.29 $gmol^{-1}$. It is an antioxidant like α-tocopherol and used in biological or biochemical applications to reduce oxidative stress (Doba, Burton, & Ingold, 1985). We used trolox because is the water soluble antioxidant so that it simplify the uptake rather than tocopherol which is oil in nature. To study the effect of antioxidants during desiccation, Araucaria embryo and mega gametophyte discs were incubated in 0.01 mM of trolox solution for about 30 minutes at 22°C dark. Incubation of samples in trolox was done before drying in order to make the embryo and mega gametophyte to absorb the trolox solution.

Viability test

Viability of embryo and mega gametophyte samples was estimated by using staining in tetrazolium. Both dried samples of trolox treated and non-treated samples of embryos and mega gametophyte were incubated in 1% solution of 2, 3, 5-triphenyl tetrazolium chloride about20-24 hours at 25-30°C in dark as recommended by (Moore, 1973). Embryos and mega gametophytes were scored as viable when they exhibited an overall carmine red staining colour.

Extraction and derivatization of primary metabolites for GC-TOF-MS analysis

Metabolome analysis performed as described previously by (Roessner et al., 2001). Samples of six pooled araucaria embryos from both with and without pre-treatment with trolox were collected at different state of moisture contents (fresh, dried and re-imbibed) and grinded to powdery before kept in -80°C under hermetic conditions. Then each grinded sample was lyophilized and approximately 10 mg were measured and transferred to 2-mL Eppendorf tube after which 400 µL and 300 µL of methanols and chloroform irrespectively were added plus 20 µL of internal standard (1 mg/ml) of ribitol then the mixture was mixed thoroughly and sonicated for 10 minutes. Thereafter, 200 µl of Milli-Q water added to the mixture followed by vortexing and centrifuging for 5 min at 13500 rpm and then methanol phase was collected in a glass vial 500 µl methanol/chloroform (1:1 v/v mix) and added to the remaining organic phase and kept on ice for 10 min. Then, 200 µl of Milli-Q water was added followed by vortexing and centrifuging for 5 min at 13500 rpm. Again the methanol phase was collected and mixed with the other collected phase. An aliquot of 100 µl of the joint phases was dried overnight by vacuum centrifugation in speedvac (35°C Savant SPD121).

Analysis of all collected samples was done by gas chromatography coupled to time of flight mass spectrometry system (GC-TOF-MS) method as previously done by (Carreno-Quintero et al., 2012). TMS derivatives were obtained via online derivatization as suggested in the paper of (Medeiros & Simoneit, 2007) and chromatography was performed in an Agilent 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus III time-of-flight mass spectrometer (Leco Instruments) using a VF-5 ms capillary column (Varian; 30 m × 0.25 mm × 0.25 µm) including a 10-m guardian column with helium as carrier gas at a column flow rate of 1 mL min^{-1}. The oven temperature program was 2 min at 70°C, followed by a 10°C min^{-1} ramp to 310°C, 5 min at 310°C, and 6 min at 70°C before the next injection. The transfer line temperature was set at 270°C. The column effluent was ionized by electron impact at 70 eV. Mass spectra were recorded at 20 scans s^{-1} within a mass-to-charge ratio range of 50 to 600 at a source temperature of 200°C. A solvent delay of 295 s was set Detector voltage was set at 1850V.

GC-MS data processing and compound identification

In the beginning, raw data were processed by using the chromaTOF software 2.0 (leco instruments) and further processed using the Metalign software (Lommen, 2009) to extract and align the mass signals. A signal to noise ratio of 2 was used. The output was further processed by the Metalign Output Transformer (METOT; Plant Research International, Wageningen) and the mass signals that where present in less than 3 RIL's where discarded. Out of all the mass signals centrotypes are formed using the MSclust program (Tikunov, Laptenok, Hall, Bovy, & de Vos, 2012). This resulted in 76 unique centrotypes (representative masses). The mass spectra of these centrotypes where used for the identification by matching to the NIST05 (National Institute of Standards and Technology, Gaithersburg, MD, USA;http://www.nist.gov/srd/mslist.htm) libraries. This identification is based on spectra similarity and comparison with retention indices calculated by using a 3rd order polynomial function (Hummel, Strehmel, Selbig, Walther, & Kopka, 2010).

Statistical analysis

In order to compare the overall variation in metabolite composition explained by differences in treatments and water states, as well as to evaluate the importance of individual axes in finding the best explanation of variance of we performed principal component analysis (PCA). The data are summarized into much fewer variables called scores which are weighted average of the original variables. The weighting profiles are called loadings. The PCA analysis is performed using the prcomp package. The calculation is based on singular value decomposition in metaboanalyst software.

RESULTS

Moisture content

At the beginning of the experiment (before drying), the moisture content of isolated mega gametophyte and embryos were not significant differ i.e. 1.72 and 2.01 g H_2O/g^{-1} DW irrespectively. The abrupt decrease of moisture content were observed after 4 hours of drying whereas the moisture content of mega gametophyte and embryos drop to 0.3 and 0.5 g H_2O/g^{-1} DW until to less than 0.1 g H_2O/g^{-1} DW after 24 hours of drying (Fig. 1). No significant difference observed between mega gametophyte and embryo in all time points of drying. However, the significant difference shown only in the comparison of moisture contents obtained at different time points in individual sample (i.e. mega gametophyte or embryo).

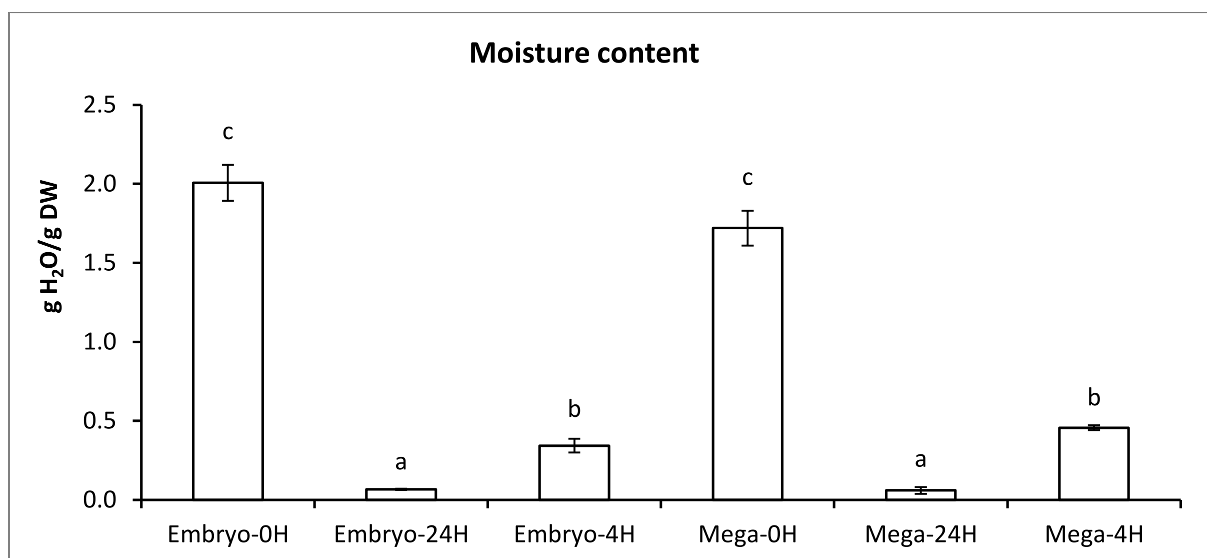

Figure 1. Moisture contents of desiccated mega gametophyte (Mega) and embryos of *A. angustifolia* seeds when dried in a different time points at 22° C, 30% RH in a closed humidity cabinet with air flow. Data are expressed in means of three replicates and bars are standard deviation. Similar letters means no significant difference

Viability test in response to antioxidants

In order to determine the effect of antioxidant on desiccated tissues, the viability of non-treated and treated samples of both mega gametophyte and embryos of *A. angustifolia* were compared. The viability was scored in tetrazolium after dehydration of the samples in different time points at 22°C, 30% RH. Before drying (0hrs) all samples were viable in tetrazolium solution (24 hours incubation, 22°C dark). Non-treated samples of both mega gametophyte and embryos were gradually lost viability as time of drying increases. The fresh mega gametophyte samples start to lose viability after 4 hours of drying and completely lost after 24 hours of drying, similar trends of viability loss was

observed in a trolox treated mega gametophyte samples (Fig 2a). Trolox treated embryos remains viable in all time points of drying while non-treated embryos completely loss viability after 4 hours of drying (Fig.2b)

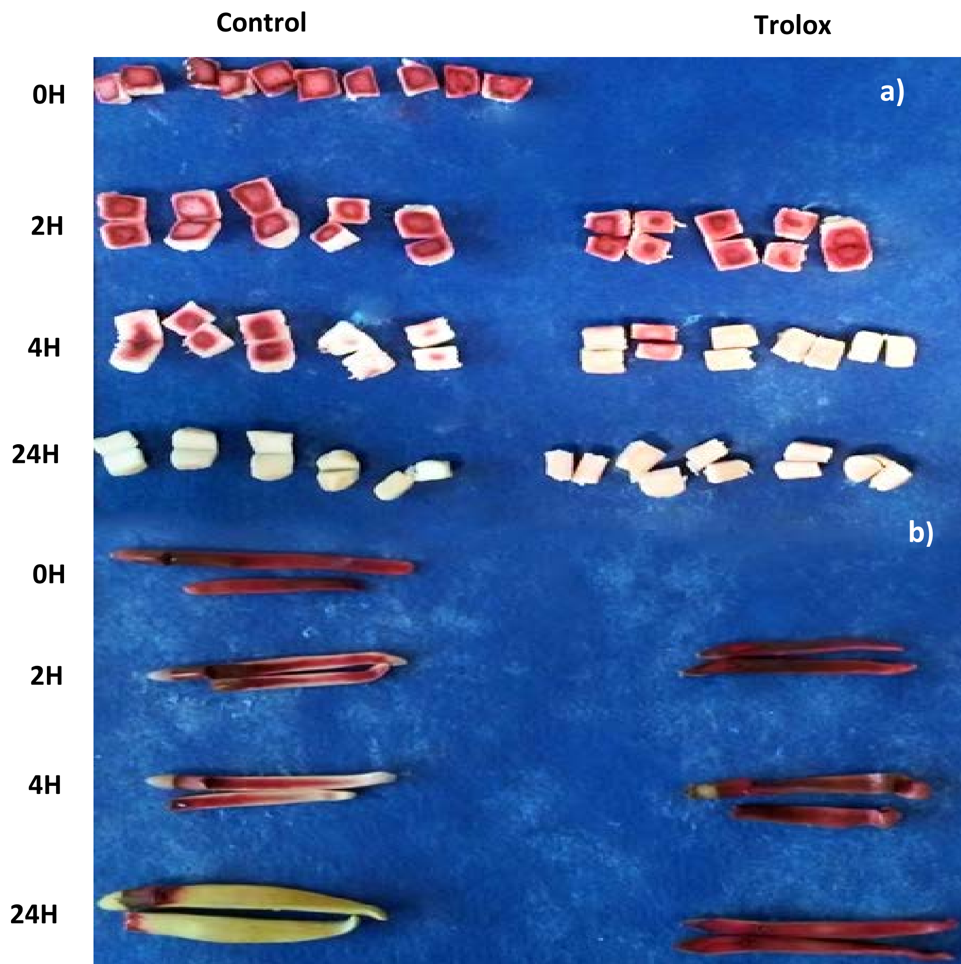

Figure 2. Variation of viability of desiccated (a) mega gametophyte and (b) embryos of *A. angustifolia* pre-treated in soluble antioxidants (trolox) and dried in different time points (0, 4 and 24 hours) at 22°C and 30% RH. The viability were determined after 24 hours of incubation the sample in 10 mL of 2, 3, 5-Triphenyl tetrazolium chloride solution at 22°C dark. They are considered as viable when staining carmine red colour after incubated for 24 hours in tetrazolium solution.

Metabolome composition of *A. angustifolia* embryos

To assess the response of metabolome due to antioxidant treatment during dehydration/rehydration cycle, 6 biological samples of embryos were collected at different water status; hydration 1(Control-Fresh), dehydration 1(Dry-control), rehydration 1(Re-Control), hydration 2 (Trolox-Fresh), dehydration 2 (Trolox-Dry), rehydration 2 (Trolox-Re imbibe) and processed by using non-biased, global metabolome technology based on chromaTOF software 2.0 and further processed using the Metalign software (Lommen, 2009) to identify the metabolites. Analysis of metabolites identification

was done by using MetaboAnalyst 3 web software. The combined softwares detected a total of 76 metabolites, of which 24 (32%) were known and 52 (68%) were non-identified (Table 1) in both non-treated and treated samples. Metabolites were categorized into classes of which non-identified were the most prevalent (68%), followed by amino acid (12%), organic acids (9%), sugars (8%) and Lastly, sugar alcohol (3%).

Table 1. Metabolites identified from a methanol tissue extract of Araucaria embryos

Amino acids	**Organic acids**	**Sugars**	**Sugar alcohols**
Alanine	Phosphoric acid	Glucose	Myo-inositol
Aspartic acid	Quinic acid	Fructose/sorbose	Galactinol
GABA	Shikimic acid	Raffinose	
Glutamic acid	Succinic acid	Sucrose	
Proline	Malic acid	Fructose/sorbose BP	
Serine	Citric acid	Kestose	
Pyro glutamic acid	Palmitic acid		
Lysine			
Ethanolamine			

Metabolomic difference during dehydration/rehydration cycle due to antioxidant treatment

Principal component analysis was used to build a comparative model of three different states of moisture contents of of *A.angustifolia* embryo with respect to treatment of trolox antioxidant. The metabolites comparison was done based on the position of the pool of the embryo in the score plot. The metabolite of re-imbibed trolox embryo and re-imbibe control embryo were similar to each other and varied for about 70.3% to samples (Dry control, Dry trolox, Fresh control and Fresh trolox). However, 10.8% variation have been observed between re-imbibe trolox embryo pool. While very small variation observation between the pools of dry control embryos, dry trolox embryos and Fresh trolox embryos.(Fig. 3)

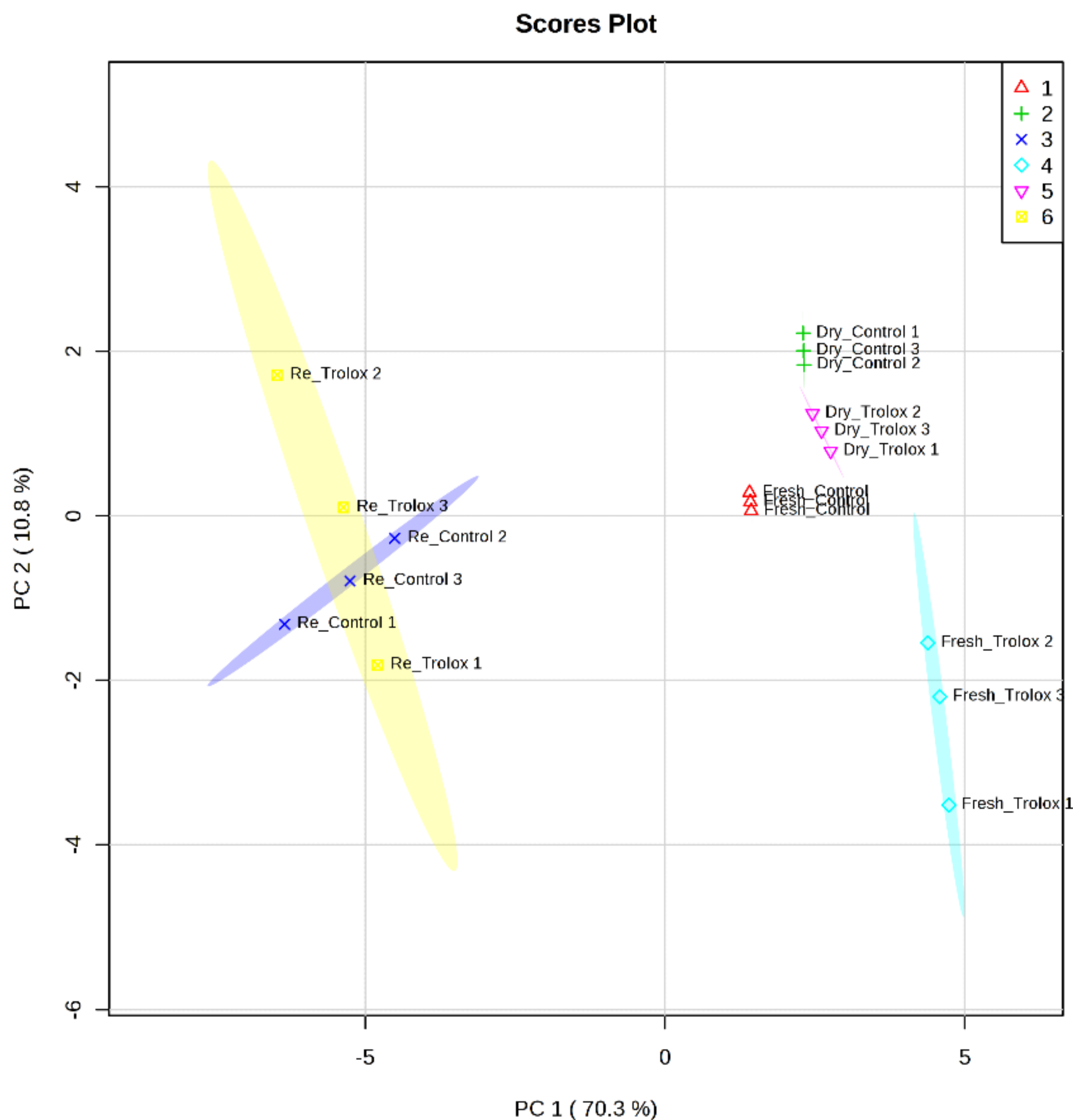

Figure 3. Principle component analysis based on metabolite profiles of *A. angustifolia* embryo in response to trolox at different water states. The distance between treatment approximates the average dissimilarity of metabolite composition between the two treatment being compared as measured by their Euclidean distance, whereas the distance between replicates approximates the dissimilarity of their metabolite content as measured by their Euclidean distance.

Response of metabolites to desiccation and antioxidants

In order to unravel metabolic changes associated with antioxidant (trolox) treatment during desiccation, we analyzed and compared the relative abundance of metabolites of *A. angustifolia*

embryos in both non-treated and treated trolox samples at three different states (hydrated, dehydrated and rehydrated). We detected 76 peaks in all treatments of embryo samples, of which only 24 have been annotated or identified. The main changes in metabolite proportion corresponded to components were associated with primary metabolism such as amino acids, organic acids, sugars and sugar alcohols as indicated in Table 1. Based on heatmap clustering (Fig. 4), the general trends of the amino acids and organic acids were increasing from fresh to re-imbibed embryoss samples in both trolox treated and non-treated samples. On the other hand sugar metabolites observed in non-treated samples were increasing during drying and decreasing during re-imbibition while In trolox treated sample the sugars metabolites were higher in fresh samples and gradual decrease during drying and final lost in re-imbibition. Despite the general trends of sugar metabolites the myo-inositol shows different trends compared to other sugar metabolites, myo-inositol were higher during re-imbibition compared to fresh and dried samples as illustrated (Fig. 4).

Sugar accumulation was varied depending on treatment whereas the fresh embryos treated with trolox have high abundances of sugar metabolites compared to non-treated embryos. Even though the trends of sugar lost during drying and re-imbibition in trolox treated sample was the similar to non-treated sample but the myo-inositol shows different pattern. Myo-inositol was increasing during re-imbibition of trolox treated sample while was lost in non-treated sample. Other metabolites, organic acid and amino acid have similar trends of increasing during re-imbibition in both trolox treated and non-treated samples. No significant changes of organic acid and amino acids have been observed in response to antioxidant application (Fig. 4).

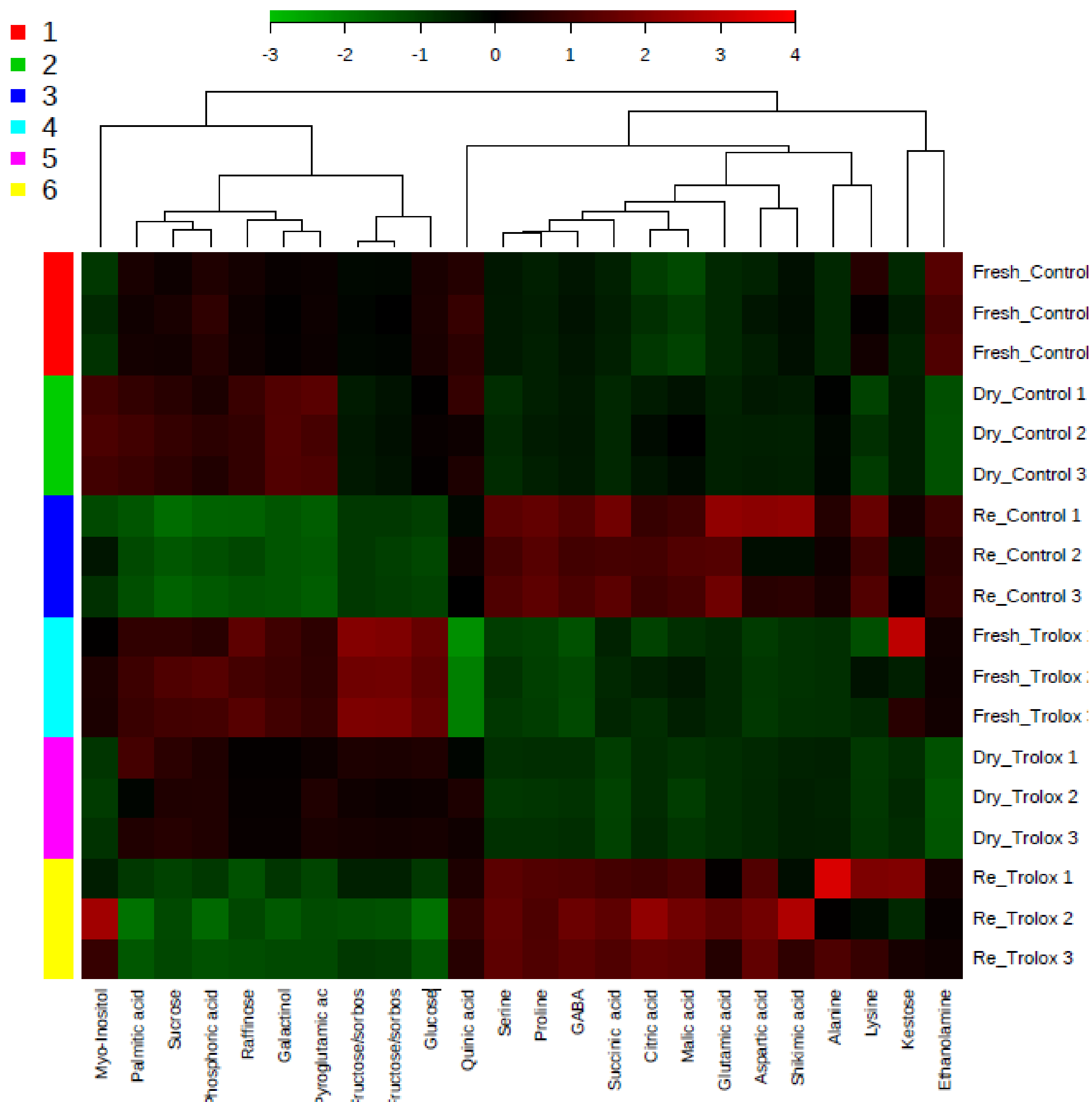

Figure 4. Clustering result shown as heatmap representation of metabolite-antioxidant correlations in response to desiccation treatment in embryo samples of *A. angustifolia*. Correlations coefficients were calculated based on Pearson`s correlation.

DISCUSSION

In this study the changes of metabolites in response to antioxidants during desiccation was investigated. The objective of this study was to giving insights into the metabolic and regulatory changes inducing DT due to antioxidant treatment. However we aimed to introduce the model of establishment of DT in recalcitrant seeds as a valuable tool to rescue the recalcitrant seeds from desiccation.

The effect of desiccation inducing cellular and metabolic damage in recalcitrant *Araucaria angustifolia* seeds has been extensively studied (Espindola, Noin, Corbineau, & Côme, 1994; Francini et al., 2006). However there is still lack of studies that correlate physiological events, such as viability or germination with metabolite changes in response to antioxidant under desiccation conditions. We hereby to present a correlation between antioxidants and metabolites changes inducing DT in recalcitrant seeds. To study metabolites changes with respect to trolox treatment and desiccation, we adopted metabolomics techniques to analyze the metabolites changes. This approach provide a detailed understanding of an organism's phenotype (Cascante & Marin, 2008; Schauer & Fernie, 2006) and disposition towards and response to environmental stresses (Urano, Kurihara, Seki, & Shinozaki, 2010). The obtained results of physiological changes and metabolites due to application water soluble antioxidants (trolox) during desiccation are discussed in this paper.

Effect of antioxidant treatment in desiccated tissue

During the storage of recalcitrant seeds water content is the critical point correlated with seed viability. It has been reported that the ability of recalcitrant seeds to survive is commonly lost during dehydration (Francini et al., 2006). However degree of sensitivity to water loss is varied among recalcitrant seeds, some being highly intolerant even in slight water loss and others withstanding a considerable degree of dehydration without any irreversible damage (P Berjak & Pammenter, 1997). Thus, for practical reasons, we defined DT as the ability of living tissue to deal with water losses below 0.1 g H_2O g^{-1} dry weight and survive the re-hydration process without permanent damage (M. J. Oliver, Tuba, & Mishler, 2000). In our experimental conditional (drying at 30% RH, 22°C), samples were considered as desiccated when it is moisture content became less than 0.1 g H_2O g^{-1} dry weight. Although the "lowest-safe moisture content" for mega gametophyte and embryos in *A. angustifolia* was found to be higher than 0.3 g H_2O g^{-1} after 4 hours of drying but viability of desiccated embryo swas completely lost after 24 hours of drying (Fig. 1) and could be rescued by pre-treatment of trolox before drying (Fig. 2). Different response in trolox has been observed between the mega gametophyte and embryo of *A. angustifolia* under desiccation. The trolox treated embryos survived in tetrazolium solution even after 24 hours of drying (Fig. 2b) while the trolox treated mega gametophyte lost its viability only after 4 hours of drying (Fig. 2a). This variation suggest that the antioxidant trolox is more effective in embryo compared to mega gametophyte. The possible reason can that the embryo is meristematic tissue so as containing other protective mechanism against cellular damage.

Overall changes of metabolites in response to desiccation and antioxidants

The common observation from the metabolomics analysis is the abundance of primary metabolites such as amino acids, organic acids, sugars and other compatible solutes. In tissues of orthodox seeds such metabolites reducing the speed of water loss from the seed and allowing the seed to synthesize compounds required for DT (Pammenter & Berjak, 1999). In our study a total of 76 metabolites were found in embryos of *A. angustifolia*, only 24 metabolites were being able to be identified (Table 1) because during GC-MS data processing and compound identification in ChromaTOF software the annotation of sucrose metabolites reach at the peak before the other 52 unknown metabolites were not being identified, therefore technically was not possible to continue with identification otherwise could damage the machine. However the PCA analysis suggest that the re-imbibe trolox embryo pool and re-imbibe control pool has little variation compared to other pools of embryo (Fig. 3). The During drying and re-imbibition most of sugars were lost to compromise with the water loss from the embryos tissue except myo-inositol, on the other hand amino acids and some of organic acid such as citric acids, succinic acid and malic acid have been increasing during re-imbibition (Fig. 4).

During dehydration of many DT species, protein breakdown and amino acid accumulation are observed (Gaff & McGregor, 1979; Tymms & Gaff, 1979). Findings from previous studies amino acids such as asparagine, arginine, glutamate, glutamine and the amino acid precursor quinate accumulated during desiccation (M. J. Oliver et al., 2011) however a decline of a proline concentration during maturation is connected with lack of DT of *machilus thunbergii* seeds (Lin & Chen, 1995). In our study, the observation of increase in amino acids during re-imbibition of *A. angustifolia* embryos suggest that the amino acids could function as compatible solutes or as mobile nitrogen reserves for the re-hydrated tissues. Despite with trolox application in embryos before drying, the metabolomics analysis did not show any significant difference in abundance of amino acids present in trolox treated embryos and non-treated embryos (Fig. 4).

According to (Dinakar & Bartels, 2013) some sugar metabolites such as sucrose, raffinose, galactinol and myo-inositol increase drastically after dehydration/rehydration cycle in most of DT seeds. These findings are not correlated with the results obtained from our study, were most of sugars found in trolox embryos were decreasing during drying and completely lost after re-imbibition except myo-inositol which was accumulated after re-imbibition. However, this observation in trolox treated sample also was different compared to non-treated embryos. The sugar metabolites were increasing during drying before completely lost during re-imbibition of non-treated embryo (Fig. 4). Although different studies hypothesized that sugars accumulated during dehydration/rehydration in

seeds, have protective functions such as replacing water on membranes and macromolecules by formation of anhydrous glass (Crowe, Carpenter, & Crowe, 1998; Hoekstra et al., 2001), vitrification of the cytoplasm (Sun & Leopold, 1997; Vertucci & Farrant, 1995), filling and stabilization of vacuoles (Farrant, 2000) and stabilization of membrane proteins (A. E. Oliver, Crowe, & Crowe, 1998). In general, the results of abundances of identified sugar metabolites obtained from our study cannot be connected with the above hypothesis.

Based on observation made in heatmap clustering graph, interesting is the increase of myo-inositol during re-imbibition of trolox treated sample compared to non-treated sample (Fig. 4). Myo-inositol metabolites is found in seeds of many different species and act as the primary source for the biosynthesis of many naturally occurring cyclitols and their derivatives in seeds of higher plants (Horbowicz & Obendorf, 1994). Cyclitols are one of the compatible solutes which are formed in a plant as a response to salt or water stress and may also serve as free-radical scavengers (Sommer, Thonke, & Popp, 1990) by activating antioxidant enzymes to counteract excess ROS. However myo-inositol have positive correlation with the accumulation of raffinose familyoligosacharide (RFO) which are mentioned to be important protective components against desiccation in seeds (Karner et al., 2004). Therefore can be concluded that protection functions of trolox in embryos may interrelated with the biosynthesis of myo-inositol which serve as primary source of solute synthesis to provide osmo-protectant in desiccated tissues. In additional myo-inositol might replace the function of antioxidant absent in recalcitrant seeds.

CONCLUSION AND FUTURE PERSPECTIVES

The ability to study mechanisms induce DT/sensitivity in a recalcitrant seeds with a combination of metabolomics techniques and in vivo physiology, create new opportunities for examining how organisms deal with desiccation stress. Based on the observation found in this research study, it is difficult to prove that the changes of protective metabolites inducing DT are influenced with antioxidant mechanisms during dehydration of seed material. Even though the survival of desiccated embryo observed in samples pre-treated with trolox but this physiological observation is not tallying with metabolites changes except myo-inositol. However the desiccated embryo pre-treated with trolox allowed to grow but it couldn`t therefore it is not yet proven that the survival desiccated embryo observed was due to application of antioxidants or increasing of myo-inositol composition. Thus we recommended further research on investigating the role of myo-inositol in induction of DT. Metabolome composition found in desiccated tissues was the same between treatments and varied only in abundance levels within the treatment, for instance amino acids were increasing during rehydration in both non-treated and treated trolox sample even though the phenotypic viability of these two samples were different; trolox samples survived while non-treated died after incubated in tetrazolium solution. On the other hand sugars and organic acids where more less the same in all moisture states of the embryos in all treatments, except myo-inositol thus the same abundances of sugar metabolites between treated and non-treated do not suggest that the sugars identified were protecting the membrane against metabolic damage. To prove and be realistic that the DT was induced in Araucaria embryos due to treatment of water soluble antioxidant (trolox) further research is needed. Identification of 52 unknown metabolites is vital importance in order to study their response to trolox during and after desiccation. Proteomics and Transcriptomic approaches should be conducted in order to unravel the effects of antioxidants in protein and genes during desiccation. DNA and lipid analysis also can give in details homeostasis of cell before and after application of antioxidants during desiccation.

ACKNOWLEDGEMENT

I would like to thank my supervisor Alexandre Marques for his close supervision and inspiration during the whole period I conduct this research study. He provided to me the guidance and ideas in both practical experiment and report writing but also I appreciate his positive criticism towards my mistakes. I would like to express my gratitude to Dr. Wilco, Ligterink and Professor Henk, Hilhorst for giving me opportunity to work on this project, their technical support towards this study and their encouragement. Final I would like to thank all Seed lab and PPH members for their support during the whole period of this research study.

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