GISIANE CAMARGO DE ANDRADE

O VIGOR EM SEMENTES DE MILHO HÍBRIDO SOB ESTRESSE PELO ENVELHECIMENTO ACELERADO É EXPLICADO PELOS COMPONENTES FISIOLÓGICOS E BIOQUÍMICOS

Dissertação apresentada ao Curso de Pós-Graduação em Produção Vegetal, na Universidade do Estado de Santa Catarina, como requisito parcial para obtenção do título de Mestre em Produção Vegetal.

Orientadora: Prof. Dra. Cileide Maria Medeiros Coelho

Coorientador: Prof. Dr. Virgílio Gavicho Uarrota

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RESUMO

ANDRADE, G. C. de. **O vigor em sementes de milho híbrido sob estresse pelo envelhecimento acelerado é explicado pelos componentes fisiológicos e bioquímicos.** 2019. 122 p. Dissertação (Mestrado) - Universidade do Estado de Santa Catarina, Centro de Ciências Agroveterinárias, Mestrado em Produção vegetal, Lages, 2019.

O aumento da demanda e das exigências por sementes de qualidade requer um maior entendimento dos mecanismos envolvidos na manifestação do vigor como um todo. Na presente pesquisa, foram realizados experimentos que permitem o avanço da compreensão das funções dos componentes fisiológicos e bioquímicos sobre o vigor de sementes de milho híbrido. No primeiro capítulo, os mecanismos de como a dinâmica dos componentes de reserva ocorre durante a germinação e a formação de plântulas de milho hibrido foram abordados. A dinâmica das reservas de sementes e a formação de plântulas dependem do genótipo e do vigor inicial avaliado pelo envelhecimento acelerado. Híbridos de maior vigor apresentam maior taxa de redução de reservas e maior mobilização de reservas para plântula, produzindo plântulas com maior massa seca, maior comprimento total, de parte aérea e raiz. No segundo capítulo, o perfil fisiológico e bioquímico de dois híbridos de milho contrastantes quanto ao nível de vigor foi acompanhando durante períodos de deterioração por envelhecimento acelerado. A maior tolerância das sementes de milho híbrido ao envelhecimento acelerado é dependente do maior teor de açúcares solúveis totais, amido e proteína solúvel total do embrião e do endosperma. Por outro lado, a maior sensibilidade ao estresse está associada a sementes com maior instabilidade de membrana e maior peroxidação lipídica no embrião e no endosperma de sementes híbridas de milho. No terceiro capítulo, foi abordada uma modelagem do vigor de sementes de milho submetidas ao envelhecimento acelerado baseado em dados de análise de infravermelho e ferramentas quimiométricas. Com base nos principais resultados, observa-se que sementes de alto vigor sofrem alterações mínimas na composição bioquímica durante o estresse pelo envelhecimento acelerado, evidenciando a relação dos compostos com o vigor das sementes, enquanto que sementes de baixo vigor são mais sensíveis ao estresse e essa menor tolerância está associada à redução dos teores de lipídios e proteínas e pelo aumento de aminoácidos, carboidratos e compostos de fósforo no embrião. Através dos resultados desta pesquisa, foram verificadas distinções entre os mecanismos bioquímicos de sementes de alto e baixo vigor, principalmente em relação à proteína solúvel total, carboidratos como açúcares solúveis totais e amido. Sementes de alto vigor demonstraram maior estabilidade de membranas celulares e tolerância ao estresse, enquanto que sementes de baixo vigor foram sensíveis ao estresse e essa sensibilidade foi associada ao aumento do metabolismo na tentativa de superação da condição adversa imposta às sementes. Assim, foi possível abrir novos caminhos para a pesquisa, sendo necessária a condução de novos estudos para identificar quais são essas proteínas e carboidratos que estão envolvidos na expressão do vigor de sementes de milho híbrido para melhorar a tolerância da cultura a condições ambientais estressantes.

Palavras-chave: *Zea mays* L. Integridade de membranas. Açúcares solúveis totais. Amido. Proteína solúvel.

ABSTRACT

ANDRADE, G. C. de. **The vigour in hybrid maize seeds under stress by accelerated ageing is explained by the physiological and biochemical components.** 2019. 122 p. Dissertation (Master degree) - State University of Santa Catarina, Agroveterinary Sciences Center, Master in Plant Production, Lages, 2019.

The increase in demand for quality seeds requires a greater understanding of the mechanisms involved in the manifestation of vigour as a whole. In the present research, experiments were performed to advance the understanding of the functions of the physiological and biochemical components on the vigour of hybrid maize seeds. In the first chapter, the mechanisms of how the dynamics of the reserve components occurs during germination and the formation of hybrid maize seedlings were addressed. The dynamics of seed reserves and the formation of seedlings depend on the genotype and the initial vigour evaluated by accelerated ageing. Hybrids with high vigour present higher seed reserves reduction rate and higher reserves mobilisation to the seedling, producing seedlings with higher dry mass higher total, shoot and root lenght. In the second chapter, the physiological and biochemical profile of two contrasting maize hybrids on the level of vigour was followed during periods of deterioration by accelerated ageing. The higher tolerance of hybrid maize seeds to accelerated ageing is dependent on the higher total soluble sugars, starch and total soluble protein content of the embryo and endosperm. On the other hand, the higher susceptibility to stress is associated to seeds with higher membrane instability and higher lipid peroxidation in the embryo and the endosperm. In the third chapter, a modeling of vigour of maize seeds submitted to accelerated ageing based on infrared analysis data and chemometric tools was discussed. Based on the main results, it is observed that high-vigour seeds undergo minimal changes in the biochemical composition during accelerated ageing stress, evidencing the relationship of the compounds with the vigour of the seeds, whereas low-vigour seeds are more sensitive to stress and this lower tolerance is associated with the reduction of lipid and protein contents and the increase of amino acids, carbohydrates and phosphorus compounds in the embryo. Through the results of this research, we distinguished the biochemical mechanisms of high and low vigour seeds, especially in relation to total soluble protein, carbohydrates such as total soluble sugars and starch. High-vigour seeds showed higher cell membrane stability and stress tolerance, whereas low-vigour seeds were susceptible to stress and this suscetibility was associated with increased metabolism in the attempt to overcome the adverse condition imposed on the seeds. Thus, it was possible to open new paths for the research, being necessary the conduction of new studies to identify which are these proteins and carbohydrates that are involved in the expression of the vigour of hybrid maize seeds to improve the tolerance of the culture to environmental stressful conditions.

Keywords: *Zea mays* L. Seed vigour. Membranes integrity. Total soluble sugar. Starch. Total soluble protein.

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1 INTRODUÇÃO

Um dos aspectos mais pesquisados recentemente na área agronômica tem sido a qualidade de sementes de diferentes espécies, especialmente a qualidade fisiológica, em função das sementes estarem sujeitas à condições adversas que proporcionam a redução do vigor. A fase mais crítica no manejo de lavouras de grandes culturas em geral é a fase de semeadura e emergência de plântulas, sendo que o sucesso dessa fase é dependente da qualidade de sementes. A utilização de sementes de qualidade é um objetivo primordial da pesquisa agronômica e das empresas produtoras de sementes. Nesse sentido, elucidar os mecanismos envolvidos nos processos de deterioração é o primeiro passo na adoção de estratégias de manejo que permitam a manutenção da qualidade de sementes.

Com o predomínio da utilização de sementes de milho híbrido e transgênico, as empresas passaram a investir cada vez mais em tecnologias, pesquisa e desenvolvimento de novas cultivares para que possam lançar híbridos que apresentem parâmetros agronômicos desejáveis, tais como produtividade, precocidade, defensividade contra pragas e doenças e estabilidade produtiva sob ampla faixa de condições ambientais. No entanto, a qualidade fisiológica de sementes não é considerada como prioridade nos programas de melhoramento, mesmo que muitos trabalhos já tenham comprovado que a germinação e o vigor podem ser aprimorados, principalmente por meio da escolha de parentais para obtenção de ganhos por heterose (SANTOS et al.; 2012; NERLING et al., 2013; OLIVEIRA et al., 2013; PRAZERES; COELHO, 2016; PRAZERES; COELHO, 2016).

Atualmente existem vários programas de controle interno e externo que visam garantir a manutenção de qualidade das sementes de milho durante todo o processo produtivo, fazendo com que a semente que o agricultor adquira esteja enquadrada dentro de padrões pré-estabelecidos pela própria empresa e pelo Ministério da Agricultura, Pecuária e Abastecimento – MAPA na Instrução Normativa n° 45 de 17 de setembro de 2013 (DIAS et al., 2015; BRASIL, 2013). Assim, o fornecimento de informações precoces que reflitam na manifestação da qualidade fisiológica são altamente desejáveis em programas de controle interno de qualidade visando reduzir gastos com tempo e custo, melhorando a eficiência dos processos num mercado de sementes altamente competitivo, como é o caso do milho (DIAS et al., 2015).

Embora inúmeros trabalhos tenham utilizado o envelhecimento acelerado para avaliar o vigor de sementes de milho, poucos utilizaram do método para acompanhar as alterações que ocorrem durante o processo em termos bioquímicos e entender o que faz um genótipo ser tolerante e outro sensível ao estresse. Além disso, é fundamental avaliar as alterações que

ocorrem nas estruturas morfológicas da semente separadamente sob condições de estresse, pois a atividade metabólica do embrião é distinta daquelas que ocorrem no endosperma.

Compreender o que acontece no metabolismo das sementes durante o processo de deterioração permite a intervenção nestes componentes bioquímicos e a prevenção da redução da qualidade das sementes. A essência da deterioração pelo envelhecimento acelerado é uma série de mudanças intrínsecas na estrutura celular e nas funções fisiológicas, físicas e bioquímicas das sementes. Entretanto, as alterações que ocorrem no envelhecimento das sementes de milho, principalmente da estrutura mais vulnerável aos processos degenerativos que é o embrião, ainda precisam ser mais detalhadas (ZHANG et al., 2007).

Diante do exposto, esta pesquisa traz o milho híbrido como objeto de estudo para tratar de uma série de aspectos que contribuem para o entendimento dos processos envolvidos na manifestação do vigor de sementes. O objetivo geral foi determinar se as alterações fisiológicas e bioquímicas durante o envelhecimento acelerado explicam as diferenças de nível de vigor existente entre os híbridos.

2 REVISÃO BIBLIOGRÁFICA

2.1 IMPORTÂNCIA DA ESPÉCIE *Zea mays* L.

O milho é o cereal mais importante do ponto de vista econômico e social cultivado no Brasil e no mundo, sendo utilizado como fonte de alimentação humana e animal para suprir parcialmente as necessidades energéticas e nutricionais, além de outras utilizações como produção de biocombustíveis, bebidas, xaropes, entre outros produtos. É uma planta anual, pertencente à família botânica Poaceae, gênero *Zea* e espécie *Zea mays* L.

O Brasil ocupa a terceira posição no ranking dos maiores produtores de milho e a segunda posição no ranking dos maiores exportadores (CONAB, 2019). Na safra 2018/19, a área cultivada com milho grão atingiu em torno de 17,3 milhões de hectares, produção de 97 milhões de toneladas e produtividade média de 5,6 toneladas por hectare (CONAB, 2019; USDA, 2019). A produção de milho é a terceira cultura que mais gera renda no Brasil, perdendo apenas para a cultura da soja e da cana-de-açúcar (SOLOGUREM, 2015).

Nesse sentido, para atender a demanda do mercado de grãos, se faz necessária a utilização de sementes de qualidade (TAVARES et al., 2016). De acordo com os dados mais atuais publicados pela Associação Brasileira de Sementes de Mudas (ABRASEM, 2019), a taxa de utilização de sementes no Brasil atingiu 92% para a safra 2017/18. Assim, percebe-se a grande preocupação dos produtores de grãos na escolha de sementes com procedência conhecida para o cultivo desse cereal.

Atualmente há uma grande preocupação quando se fala da produção de alimentos para suprir as necessidades da população mundial, que deverá ter um crescimento na ordem de 34,9 % até o ano de 2050, alcançando 9,5 bilhões de pessoas (ONU, 2012; SAATH; FACHINELLO, 2018). Nesse sentido, as sementes de milho tem um papel fundamental, pois está diretamente atrelada à produção do grão, impactando também os setores de bovinocultura, suinocultura e avicultura e, consequentemente, a produção de alimentos (OLIVEIRA NETO, 2008). A semente deve ser considerada um dos principais investimentos da agricultura para que seja possível atender as demandas em termos de quantidade e qualidade de grãos (HAMPTON et al., 2016; SAATH; FACHINELLO, 2018).

2.2 ASPECTOS DAS CULTIVARES DISPONÍVEIS NO MERCADO

A escolha de cultivares é um fator preponderante para o sucesso ou falha da produtividade da lavoura (COELHO et al., 2004). O milho é cultivado de norte a sul, leste a oeste do Brasil, por pequenos, médios e grandes produtores. A semente pode ser considerada como um meio de transporte das tecnologias e informações que foram incorporadas à ela

durante o melhoramento genético para o campo (KRZYZANOWSKI, 2009). No entanto, o custo da semente é relativamente alto, variando em função da escolha do material a ser semeado, impactando consideravelmente no custo de produção da lavoura. De acordo com a Companhia Nacional de Abastecimento que efetuou a análise dos custos de produção da cultura entre os anos-safra de 2007 a 2017, a semente pode impactar em até 25% do custo de produção (CONAB, 2018).

No levantamento de safra mais atualizado pelo Ministério da Agricultura, Pecuária e abastecimento (MAPA), na safra de 2016/17 foram disponibilizados 315 materiais distintos no mercado, sendo variedades de polinização aberta (VPAs), híbridos simples (HS), simples modificados (HSm), duplos (HD), triplos (HT) e triplos modificados (HTm) (PEREIRA FILHO; BORGHI, 2016; MAPA, 2016). Desse total, 67,9 % das cultivares disponibilizadas no mercado apresentam algum evento de transgenia e as demais são convencionais (PEREIRA FILHO; BORGHI, 2016; MAPA, 2016). No entanto, aproximadamente 90% das cultivares utilizadas para o cultivo do cereal no Brasil apresentam um ou mais eventos tecnológicos de transgenia (CÉLERES, 2017). Dessa forma, observa-se o grande predomínio e preferência por cultivares transgênicas

O cultivo de milho por grandes produtores normalmente visa a obtenção de patamares elevados de produtividade, acima da média de 5,6 toneladas por hectare (CRUZ et al., 2011). Nesse sentido, os híbridos simples e triplos tem ocupado maior área cultivada devido ao seu maior potencial produtivo (PEREIRA FILHO; BORGHI, 2016). Independente do híbrido a ser escolhido, uma característica que está intimamente relacionada com o aumento da demanda por sementes é a necessidade de adquirir a semente híbrida toda a safra. Já é popularmente sabido que a redução da produtividade em função da utilização da segunda geração dessas sementes provoca reduções de 15 a 40% da produtividade final da lavoura (FRITSCHE-NETO; MÔRO, 2015).

O primeiro evento transgênico foi liberado em agosto de 2007 para o cultivo pelos agricultores na safra de 2008 (DUARTE et al., 2009; MORAIS; BORÉM, 2015). Desde então, a aceitação e o aumento da utilização dessas cultivares vem crescendo safra após safra, com cerca de 20 eventos transgênicos aprovados atualmente. Esses eventos transgênicos envolvem a tolerância de herbicidas tais como glifosato e glufosinato de amônio, entre outros, a resistência à pragas da Ordem Lepidóptera ou ainda a piramidação dessas tecnologias.

Diante desse cenário, as empresas produtoras de sementes de milho tem apostado no desenvolvimento de cultivares com características de precocidade, produtividade, estabilidade produtiva em diferentes condições edafoclimáticas, além da defensividade contra pragas e

doenças. Como o custo do investimento em sementes é alto, disponibilizar sementes de qualidade é essencial para essas empresas para mantê-las competitivas, pois o mercado está tornando os consumidores cada vez mais exigentes nesse contexto.

2.3 ATRIBUTOS RELACIONADOS À QUALIDADE DE SEMENTES

2.3.1 Qualidade de sementes

O sucesso ou falha de uma lavoura é dependente da qualidade da mesma e para obter estandes uniformes, com populações de plantas adequadas, se faz necessária a utilização de sementes de qualidade (FRANÇA-NETO et al., 2010; FINCH-SAVAGE; BASSEL, 2015).

A qualidade de sementes é o somatório ou ainda a interação entre os atributos genéticos, físicos, fisiológicos e sanitários, sendo que cada um desses fatores conferem características essenciais durante a germinação e o estabelecimento de plântulas (POPINIGIS, 1985; CORBINEAU, 2012; MARCOS-FILHO, 2015). Portanto, para que uma semente possa ser considerada de qualidade, ela deve possuir características que contemplem todos esses atributos.

A qualidade genética envolve a pureza varietal da semente e deve manifestar as características que foram incorporadas à ela durante o melhoramento genético (POPINIGIS, 1977; MARCOS-FILHO; 2015), tais como precocidade, produtividade, defensividade contra pragas e doenças, além das características transgênicas. É através da manutenção da pureza genética que o produtor tem garantia de estar semeando em sua lavoura a semente da cultivar que ele adquiriu.

A qualidade física envolve as propriedades de umidade da semente, a presença de danos mecânicos e injúrias provocadas por insetos, a presença de sementes de outras espécies e material inerte, o peso de mil sementes, além da uniformidade do beneficiamento dos lotes (POPINIGIS, 1985; MARCOS-FILHO; 2015).

A qualidade fisiológica, como tema do presente estudo, é o conjunto de características relacionadas às funções vitais da semente, tais como a germinação e o vigor (POPINIGIS, 1985; MARCOS-FILHO; 2015). O máximo potencial em termos de qualidade fisiológica é alcançado na maturidade fisiológica, que corresponde ao momento da formação da sementes em que ela acumula o máximo de massa seca e perde a conexão direta com a planta que a originou e passa a responder conforme o ambiente em que ela está (POPINIGIS, 1985; MARCOS-FILHO, 2015). Sendo assim, logo após a maturidade fisiológica, a semente fica propensa ao declínio da qualidade, sendo necessária a adoção de estratégias de manejo que minimizem a velocidade das

mudanças degenerativas de origem fisiológicas e bioquímicas, uma vez que ela não pode ser evitada (MARCOS-FILHO, 2015).

Por fim, a qualidade sanitária está relacionada à presença de patógenos como fungos, bactérias ou vírus, que possam estar infectando ou infestando as sementes (POPINIGIS, 1985; MARCOS-FILHO, 2015).

2.3.2 Vigor e sua relação com a deterioração de sementes

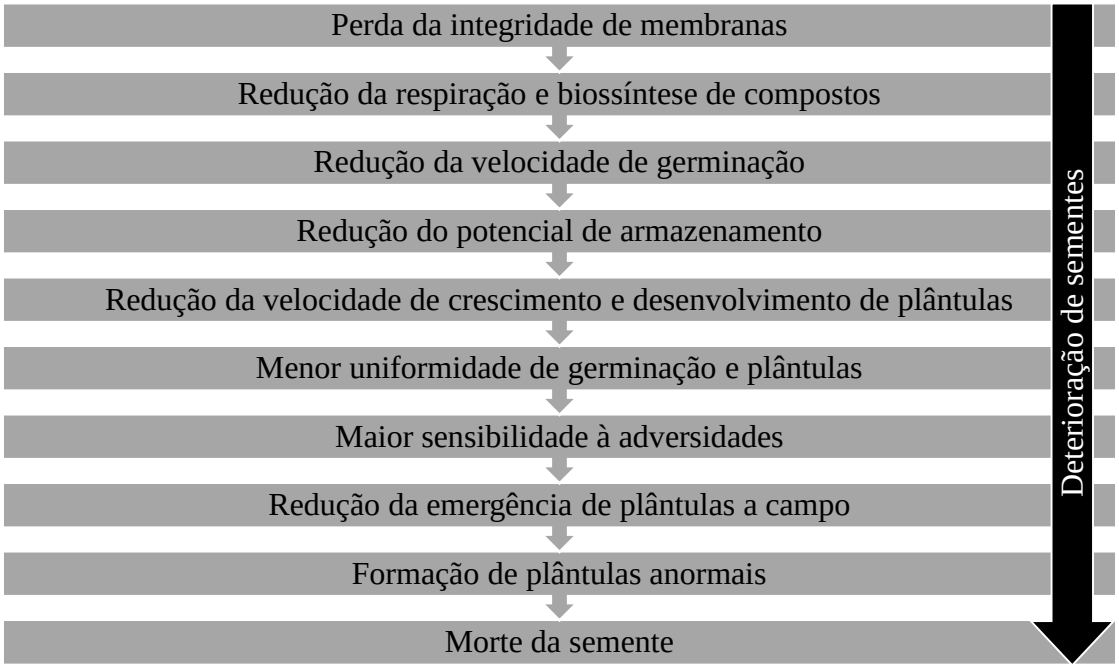
O vigor é uma característica complexa e intrínseca das sementes que confere a capacidade de germinação e formação de plântulas normais rápida e uniforme sob amplas condições edafoclimáticas, bióticas ou abióticas (RAJJOU et al., 2012; FINCH-SAVAGE; BASSEL, 2015; MARCOS-FILHO, 2015). Nesse sentido, a área de tecnologia de sementes tem avançado rapidamente no desenvolvimento e na aplicação de novos métodos de avaliação que sejam capazes de diferenciar a qualidade fisiológica dos lotes, principalmente quando a germinação entre eles é semelhante (MARCOS-FILHO, 2015).

A avaliação do vigor das sementes é essencial para identificar o grau de deterioração de lotes em sua fase inicial. Dessa forma, pode-se tomar decisões acerca do descarte de lotes ou até mesmo de mudanças de estratégias quanto ao manejo das sementes. Sabendo-se que é impossível evitar que a deterioração ocorra ou reverter o processo, é essencial adotar práticas que visem minimizar a evolução da deterioração (DELOUCHE, 1963).

Conceitualmente, a deterioração de sementes pode ser entendida como alterações bioquímicas, morfológicas e fisiológicas que ocorrem internamente quando às sementes são expostas a condições adversas que provocam a degeneração gradual até a perda total da viabilidade (DELOUCHE e BASKIN 1973, BEWLEY et al., 2013; MARCOS-FILHO, 2015). Essas alterações são visualizadas através das manifestações externas, como por exemplo a queda da velocidade de germinação e emergência, o surgimento de plântulas anormais e declínio da capacidade germinativa, resultando em lotes com menor percentual de germinação (MARCOS-FILHO, 2015). Por outro lado, internamente estão ocorrendo reações bioquímicas que provocam a redução da respiração e da síntese de ATP, alterações em atividades enzimáticas e no metabolismo de reservas, produção de radicais livres, entre outros (MARCOS-FILHO, 2015).

A sequência de alterações que ocorrem quando uma semente está em processo de deterioração foi proposta por DELOUCHE e BASKIN (1973) (Figura 1).

Figura 1- Sequência provável de alterações fisiológicas e bioquímicas durante o processo de deterioração de sementes.



Fonte: Elaborado pela autora, 2019. Adaptado de DELOUCHE e BASKIN (1973) e MARCOS-FILHO (2015).

Uma das primeiras alterações é a perda de integridade de membranas celulares e, consequentemente, o aumento da permeabilidade e a perda de eletrólitos para o meio externo, tais como proteínas, açúcares, potássio, cálcio, entre outros (ALVES et al., 2004, BEWLEY et al., 2013; MARCOS-FILHO, 2015). Isso ocorre porque durante o processo de deterioração ocorrem reações químicas que provocam a liberação de substâncias tóxicas, tais como malondialdeído, espécies reativas de oxigênio como peróxido de hidrogênio, radicais superóxidos e hidroxilas, oxigênio singleto, entre outros. Os radicais livres e os compostos tóxicos promovem a peroxidação de lipídios e são considerados principais causadores da deterioração de membranas (PRIESTLEY et al., 1985; COOLBEAR, 1995). Inúmeros trabalhos podem ser encontrados na literatura utilizando a metodologia do teste de condutividade elétrica para determinar o vigor de sementes, pois ele é considerado um dos mais sensíveis na detecção dos processos de deterioração em sua fase inicial através da medição indireta da integridade de membranas celulares.

Além disso, outras alterações resultantes do processo de deterioração de membranas é a redução da atividade respiratória e o comprometimento do ciclo de Krebs, da cadeia respiratória e da fosforilação oxidativa, provocando um decréscimo na produção de energia química (trifosfato de adenosina – ATP) para a manutenção do metabolismo. Ocorrem também a perda da compartimentalização celular, redução da atividade enzimática e da síntese de proteínas. Em

estágios mais avançados, ocorre o surgimento de plântulas anormais devido à morte de tecidos meristemáticos (MARCOS-FILHO, 2015).

2.3.3 Principais testes de vigor de sementes e plântulas

De acordo com a classificação proposta por McDONALD (1975), os testes de vigor podem ser físicos, fisiológicos, bioquímicos ou ainda de resistência. Os testes físicos são aqueles cujas metodologias avaliam aspectos morfológicos ou físicos que possam estar relacionado ao vigor das sementes, como por exemplo tamanho de sementes, peso unitário, densidade, coloração e mais recentemente, análises de raio X e de imagens de sementes (MARCOS-FILHO, 2015).

Os testes fisiológicos são aqueles relacionados com o vigor de sementes e plântulas, tais como primeira contagem de germinação, velocidade de germinação e emergência de plântulas, mobilização de massa seca da semente para a plântula e crescimento de plântulas (MARCOS-FILHO, 2015). Esses testes são frequentemente utilizados na área de tecnologia como forma de determinar a formação de plântulas normais sob condições de estresse, ou seja, a manifestação visual ou externa do vigor.

Por outro lado, os testes bioquímicos são aqueles que avaliam as alterações bioquímicas que ocorrem internamente e que podem estar associadas ao vigor. São exemplos de testes classificados como bioquímicos o teste de respiração, tetrazólio, condutividade elétrica, lixiviação de potássio, determinação de atividade enzimática e do teor de componentes de reserva, entre outros (MARCOS-FILHO, 2015).

Por fim, os testes de resistência são aqueles onde as condições as quais as sementes são expostas não são consideradas como ideais. Dessa forma, lotes de sementes que toleram essas condições de estresse que foram impostas à eles são considerados vigorosos. São exemplos mais comuns de testes dessa categoria o teste de frio, envelhecimento acelerado, imersão em soluções tóxicas, entre outros (MARCOS-FILHO, 2015).

Para melhor entender a característica de vigor de sementes de milho híbrido e tentar compreender os mecanismos de causas e efeitos do envelhecimento das sementes, no presente estudo foram utilizados métodos físicos, fisiológicos, bioquímicos e de resistência para a avaliação mais aprofundada e confiável.

2.4 ESTRUTURAS E COMPOSIÇÃO QUÍMICA DE SEMENTES DE MILHO

2.4.1 Estruturas morfológicas das sementes

As sementes ou os grãos de milho, considerados botanicamente como um fruto seco chamado cariopse, são formados por quatro estruturas físicas: endosperma, embrião, pericarpo e pedicelo ou ponta do grão (BEWLEY et al., 2013).

O embrião representa em torno de 11,5 % do total da semente e é a estrutura resultante do processo de dupla fecundação da oosfera, ou seja, da célula feminina haploide (n) com uma célula masculina haploide, chamada célula espermática (n), formando uma estrutura diploide (2n) (FERREIRA; BORGHETTI, 2004; CARVALHO; NAKAGAWA, 2012; BEWLEY et al., 2013; MARCOS-FILHO, 2015).

Em sementes de espécies pertencentes à classe das monocotiledôneas como é o caso do milho, o embrião é considerado uma planta em miniatura, formado pelo eixo embrionário, que contém as estruturas já diferenciadas de coleorriza, radícula, mesocótilo, plúmula e coleóptilo, além do cotilédone único, também chamado de escutelo (BEWLEY et al., 2013). Essa é a estrutura fundamental para a propagação da espécie, uma vez que ela dará origem a uma nova planta.

O endosperma representa mais de 82 % do total da semente e é a estrutura originada a partir da união da célula espermática (n) com os núcleos polares (2n), formando uma estrutura triploide (3n) (FERREIRA; BORGHETTI, 2004; CARVALHO; NAKAGAWA, 2012; BEWLEY et al., 2013; MARCOS-FILHO, 2015). Essa estrutura possui a função de fornecer os nutrientes necessários para o crescimento e desenvolvimento do embrião para a formação de plântulas. A maioria das células no endosperma é não-viva, sendo que apenas a camada mais externa, chamada camada de aleurona permanece viável. Essa camada não possui a função de armazenar nenhuma reserva, mas é responsável pela produção e liberação de enzimas para a hidrólise e mobilização das reservas (BEWLEY et al., 2013).

As outras duas estruturas restantes correspondem representam pouco do total da semente, mas possuem funções igualmente importantes às demais. O pericarpo ou casca representa 5,3 % do total da semente e é a barreira que protege contra o contato direto entre as estruturas internas da semente e o ambiente externo (BEWLEY et al., 2013).

O pedicelo ou ponta da semente representa apenas 0,8 % do total e é a estrutura morfológica localizada na região de inserção do grão ou semente ao sabugo. No momento da maturidade fisiológica, as células do pedicelo são pressionadas devido ao máximo acúmulo de massa no grão, que faz com que essas células fiquem enegrecidas (DAYNARD; DUNCAN,

1969; DIAS, 2001). Assim, um dos métodos de identificação do ponto de maturidade fisiológica em sementes de milho é a formação da camada negra na região do pedicelo (DAYNARD; DUNCAN, 1969).

2.4.2 Composição química da semente e suas funções

A semente ou grão de milho são utilizados na alimentação humana devido à grande quantidade de energia fornecida pelos teores de amido e lipídios presentes no grão. O peso unitário das sementes de milho normalmente variam entre 200 a 300 mg, sendo que as estruturas que a compõe tais como endosperma, embrião, pericarpo e pedicelo diferem entre si quanto a composição química. A composição de cada fração e da semente inteira está apresentada na Tabela 1.

Tabela 1 – Composição química das sementes de milho em porcentagem.

Frações	Semente	Amido	Proteína	Lipídios	Açúcares	Cinza
			%			
Endosperma	82,3	86,4	9,4	0,8	0,6	0,3
Embrião	11,5	8,2	18,8	34,5	10,8	10,1
Pericarpo	5,3	7,3	3,7	1,0	0,3	0,8
Pedicelo	0,8	5,3	9,1	3,8	1,6	1,6
Semente	99,9	71,5	10,3	4,8	2,0	1,4

Fonte: Elaborado pela autora, 2019. Adaptado de TOSELLO, 1987 e CARVALHO; NAKAGAWA, 2012.

O endosperma é considerado como estrutura de reserva da semente, que fornece os nutrientes necessários para o crescimento e desenvolvimento do embrião durante o processo de germinação (BEWLEY et al., 2013; MARCOS-FILHO, 2015). Essa estrutura é formada principalmente por amido (86,4 %) e proteína (9,4 %) (CARVALHO; NAKAGAWA, 2012).

O amido é um polissacarídeo formado pela união de amilose e amilopectina (COPELAND; McDONALD, 2012) que sofre ação das enzimas α e β-amilase para liberação de compostos mais simples, tais como maltose e glicose. A glicose pode ser prontamente utilizadas nos processos respiratórios para transformação em energia química, enquanto que a maltose sofre ação de outra enzima, a maltase, dando origem à sacarose, que pode ser então transportadas para o embrião (BEWLEY et al., 2013; MARCOS-FILHO, 2015).

As maior fração de proteínas do milho são chamadas de prolaminas (zeínas), proteínas ricas em aminoácidos prolina e glutamina, que envolvem os grânulos de amido dentro das células do endosperma e constituem 85% da fração proteica no milho (BEWLEY et al., 2013).

A camada mais externa do endosperma, chamada camada de aleurona, possui uma grande função no processo de germinação de sementes. Com a absorção de água pelas sementes, a camada de aleurona produz uma enzima hidrolítica importante chamada α-amilase em reposta

ao ácido giberélico, que promove a degradação do amido e liberação de açúcares solúveis para serem utilizados para o crescimento e desenvolvimento do embrião (BEWLEY et al., 2013; MARCOS-FILHO, 2015). Além disso, é na camada de aleurona que estão presentes os carotenoides que conferem a coloração dos grãos ou sementes (PAES, 2006).

O embrião compreende aproximadamente 11,5% do total da semente, sendo composto principalmente por lipídios (34,5 %), proteínas (18,8 %) e açúcares (10,8 %) (CARVALHO; NAKAGAWA, 2012). Essa é a única estrutura viva da semente de milho. Apesar de apresentar um teor proteico alto rico em metionina e cisteína, as proteínas são pobres em lisina e triptofano, que são considerados aminoácidos essenciais para a nutrição animal e do homem, sendo fundamental complementar as rações ou a alimentação com outras fontes proteicas (PAES, 2006).

2.4.2.1 Principais funções e alterações nos componentes químicos em condições de estresse

Em condições de estresse como o envelhecimento acelerado, por exemplo, apesar das sementes não entrarem em contato direto com a água, o grau de umidade aumenta gradualmente com o aumento do período de exposição devido à característica de higroscopicidade que as sementes apresentam. Isso significa que as sementes possuem a capacidade de elevar ou reduzir o grau de umidade conforme o ambiente que ela está para se aproximar da condição de equilíbrio com o ambiente externo (MARCOS-FILHO, 2015). Essa umidade por si só é suficiente para ativar alguns mecanismos bioquímicos dependentes da água. Assim, os componentes de reserva tem importância fundamental para auxiliar no entendimento do vigor, pois desempenham funções essenciais no estresse e não somente como fonte energética durante o processo de germinação. A seguir serão abordadas as funções componentes químicos majoritários em situações de estresse.

Segundo MARCOS-FILHO (2015), os efeitos provocados pelos processos de deterioração podem ser classificados como fisiológicos ou bioquímicos. Nesse sentido, o uso do teste de envelhecimento acelerado tem potencial para possibilitar a compreensão das alterações bioquímicas envolvidas nos mecanismos de deterioração durante o envelhecimento das sementes (DELOUCHE; BASKIN, 1973; ALVES et al., 2004; ZHANG et al., 2008) e para associar com a manifestação fisiológica do vigor através da formação de plântulas normais (DELOUCHE; BASKIN, 1973).

O envelhecimento acelerado de sementes sob condições de alta temperatura (> 40 °C) e alta umidade relativa é consistente com o envelhecimento em condições naturais, mas em uma taxa mais alta (DELOUCHE; BASKIN, 1973). A velocidade do processo de envelhecimento

da semente depende da capacidade que ela apresenta em resistir a processos degenerativos que ocorrem nessas condições, bem como da eficácia dos seus mecanismos de proteção (BALEŠEVIĆ-TUBIĆ, 2012).

Um dos fatores que influenciam na deterioração das sementes e, consequentemente, no declínio do vigor é a composição química das sementes (BALEŠEVIĆ-TUBIĆ, 2012; MARCOS-FILHO, 2015). Os componentes químicos presentes no tecido de reserva de sementes podem ser hidrolisados enzimaticamente a partir de compostos complexos (por exemplo, amido, proteínas, lipídios) a compostos simples (por exemplo, glicose, aminoácidos, ácidos graxos, glicerol) para serem mobilizados em direção ao embrião durante a germinação, para respiração e manutenção dos tecidos vivos em condições de estresse ou ainda para a síntese de compostos de reparo de estruturas danificadas pela deterioração (BEWLEY et al., 2013; HAN et al., 2017).

2.4.2.2 Lipídios

A oxidação de lipídios e ácidos graxos é uma das primeiras reações que ocorrem em situações de estresse, produzindo radicais livres que provocam reações degenerativas em cadeia, afetando negativamente não somente esse componente, como também proteínas, carboidratos e ácidos nucleicos (McDONALD, 1999; ALVES et al., 2004). Além disso, o acúmulo de ácidos graxos livres promove redução do pH celular, prejudicando a manutenção da integridade de proteínas e da atividade enzimática (MARCOS-FILHO, 2015).

Os lipídios e ácidos graxos encontrados nas sementes podem ser utilizados como indicadores de qualidade de sementes ou do grau de deterioração das sementes, principalmente devido à instabilidade físico-química desses componentes (BALEŠEVIĆ-TUBIĆ, 2012, MARCOS-FILHO, 2015). Vale ressaltar que os ácidos graxos insaturados são mais propensos aos processos de deterioração. O embrião das sementes de milho é composto por mais de 60 % de ácido linoleico e 24 % de ácido oleico, ou seja, mais de 80 % da composição dos ácidos graxos presentes no embrião de sementes de milho é insaturado, com grande vulnerabilidade à deterioração (BEWLEY et al., 2013).

Como a maior parte dos lipídios nas sementes de milho está concentrada no embrião, ele se torna mais vulnerável a ocorrência de processos degenerativos e à perda de viabilidade dos tecidos. Ainda, não somente os lipídios armazenados sofrem peroxidação, os fosfolipídios presentes em membranas celulares também são negativamente afetados (MARCOS-FILHO, 2015). Portanto, utilizar métodos para determinar o nível de peroxidação lipídica em embriões de sementes de milho é fundamental para o entendimento do vigor.

2.4.2.3 Proteínas

Os processos deletérios provocados pelo estresse produz efeito negativo direto na atividade enzimática, na produção de novas enzimas e na estrutura de proteínas, afetando consequentemente, as suas funções (McDONALD, 1999, BEWLEY et al., 2013; MARCOS-FILHO, 2015). Em condições de temperaturas elevadas (> 40°C), como no caso do envelhecimento acelerado de sementes, as principais alterações a nível proteico está relacionada com a desnaturação desse componente (MARCOS-FILHO, 2015).

Alguns pesquisadores citam a deterioração de membranas de organelas celulares como retículo endoplasmático e do complexo golgiense, que são provocadas pela peroxidação de lipídios e seus produtos, impactam negativamente na síntese proteica, reduzindo os níveis desse composto nas sementes (BRACCINI et al., 2001).

Outra forma de identificação da deterioração é por meio do acompanhamento da atividade de enzimas antioxidantes, como a superóxido dismutase (SOD), catalase (CAT), as peroxidases, entre outras, que possuem a função de combater e remover os radicais livres e produtos tóxicos das células (BALEŠEVIĆ-TUBIĆ, 2012; BEWLEY et al., 2013; MARCOS-FILHO, 2015). Em estudo realizado por SPINOLA et al. (2000) foram avaliados os perfis eletroforéticos das enzimas fosfatase ácida e peroxidase durante períodos diferentes do teste de envelhecimento acelerado de sementes de milho. Os autores concluíram que a avaliação da atividade enzimática é capaz de indicar o efeito de deterioração provocado pelo estresse.

As principais alterações que ocorrem nesse componente estão relacionadas com o decréscimo dos níveis proteicos e da síntese de novas proteínas, desnaturação e perda de funções e acréscimo dos níveis de aminoácidos livres (MARCOS-FILHO, 2015). Praticamente todas as reações bioquímicas que ocorrem nos seres vivos dependem de proteínas através da ação catalisadora por enzimas. Assim, alterações nesse componente podem trazer informações valiosas quanto ao vigor de sementes, tendo em vista que sementes de baixo vigor tem seus processos bioquímicos prejudicados quando expostas à situações adversas.

2.4.2.4 Carboidratos

Além de serem a principal fonte de energia para a manutenção dos processos metabólicos, outro aspecto importante associado aos carboidratos é a sua função no processo de transição entre semente seca e semente hidratada. Após a maturidade fisiológica, as sementes perdem água e passam por modificações para tolerar a desidratação e posterior reidratação. Os carboidratos, especialmente os oligossacarídeos tais como rafinose, estaquiose e verbascose,

estão intimamente envolvidos com essa tolerância, com a função de manter os espaços entre as os fosfolipídios e proteínas de membrana celular (BEWLEY et al., 2013; MARCOS-FILHO, 2015).

Ao perder água, as membranas saem do estado líquido-cristalino para um estado menos fluido, chamado estado vítreo (BEWLEY et al., 2013; MARCOS-FILHO, 2015). Isso só é possível porque os carboidratos presentes nas células se ligam ao fósforo da camada fosfolipídica, minimizando a desestruturação e mantendo a organização das membranas (BEWLEY et al., 2013; MARCOS-FILHO, 2015). Essa função dos carboidratos é fundamental para manter a compartimentalização celular e a reorganização de membranas após a reidratação. Caso contrário, poderia ocorrer o empacotamento de membranas com a retomada da entrada de água na semente.

Se essa reorganização não ocorre de forma eficiente, ocorre o extravasamento de solutos e a redução do vigor das sementes. Assim, a redução dos níveis de oligossacarídeos podem afetar o grau de tolerância à estresses, especialmente pela redução da proteção contra a perda de integridade de membranas proporcionada pelos açúcares. Em situações de estresse em geral, ocorrem decréscimos nos níveis de açúcares solúveis e amido, resultando em menor disponibilidade de substratos para os processos respiratórios, fazendo com que a semente entre em colapso metabólico, comprometendo assim a manutenção da viabilidade (McDONALD, 1999; MARCOS-FILHO, 2015). Alguns pesquisadores da atualidade tem dedicado esforços no estudo de oligossacarídeos da família da rafinose e sua relação com o vigor de sementes (EGERT et al., 2015; ZHANG et al., 2017).

Como a deterioração promove o decréscimo na atividade de enzimas como a α-amilase e, por consequência, dos teores de amido e açúcares solúveis totais, o estudo comparativo entre o comportamento desses componentes em sementes de alto e baixo vigor tende a trazer informações relevantes quanto aos mecanismos envolvidos na expressão do vigor, especialmente em espécies ricas em carboidratos, como é o caso do milho.

2.4.2.5 DNA mitocondrial e nuclear

O DNA mitocondrial e nuclear também são componentes vulneráveis do processo de deterioração, pois os mecanismos de reparo sofrem também são afetados negativamente em situações de estresse. Além disso, a síntese proteica de reserva ou metabolicamente ativas (enzimas) também é prejudicada (ABDUL-BAKI, 1980). Como consequência, ocorre o impedimento da formação de novas estruturas ou a limitação do crescimento normal de estruturas já formadas.

Diante do exposto, determinar qual ou quais os fatores que tornam um genótipo mais tolerante do que outro em condições adversas faz com que essa característica possa ser acompanhada desde as primeiras etapas dos programas de melhoramento genético. Por outro lado, para identificar o que torna uma semente sensível ou tolerante à uma determinada condição causando o declínio da qualidade da semente são questões que ainda não foram completamente elucidadas pela pesquisa. Para que seja possível melhorar a tolerância de cultivares à estresses, é necessário entender os mecanismos responsáveis que estão envolvidos nos processos deterioração de sementes.

3 CHAPTER 1 - SEED RESERVES REDUCTION RATE AND RESERVES MOBILISATION TO THE SEEDLING EXPLAIN THE VIGOUR OF HYBRID MAIZE SEEDS

3.1 ABSTRACT

The understanding of the mechanisms of how the reserve components dynamics occurs during germination and seedling formation is determinant for the contribution to the advancements of seed technology. The aims of this study were: (I) to verify which accelerated ageing temperature is the most effective in the separation of the vigour levels of hybrids; (II) to evaluate the dynamics of the reserves during germination and the seedling process in maize seeds with different vigour levels; (III) to perform correlations between the characteristics evaluated and (IV) to use multivariate analysis tools to identify the characteristics that contributed most to the vigour of hybrid maize. Seeds of seven cultivars were submitted to the tests of moisture degree, germination rate, accelerated ageing test, one thousand seed weight, total seed length, shoot and root length, dry matter of seed and seedling, remaining dry matter in the endosperm, reduction of seed reserves, conversion efficiency of seed reserves and reserves mobilisation rate to the seedling using the completely randomized statistical design. Significant positive correlations were observed between the rates and vigour by accelerated ageing, total seed length, root and shoot length, dry matter of seedling, reduction of seed reserves, and a significant negative correlation of the rates with the variable remaining dry matter in the endosperm. The dynamics of seed reserves and seedling formation depends on the genotype and the initial vigour evaluated by accelerated ageing. Higher vigour hybrids present higher seed reserves reduction rate and higher reserves mobilisation to the seedling, producing seedling with higher dry matter of seedling, higher total seed length, shoot and root length, regardless of seed size. Seed reserves reduction rate and reserves mobilisation to the seedling explain the vigour of hybrid maize seeds and can be used in breeding programs aimed at the selection of cultivars with high physiological quality.

3.2 INTRODUCTION

Maize or corn (*Zea mays* L.) is one of the most cultivated cereal crops and the most popular energetic source for humans and animals around the world, with approximately 1,1 billion tons produced annually (USDA, 2019). To achieve higher production and yield, there are many variables that can influence the plant development. The role of seeds is one of the most important factor in the establishment of crops due to seed quality, which includes genetic, physical, sanity and physiological attributes, is essential for successful germination and seedling growth (MARCOS FILHO, 2015).

For maize crops, uniformity of sowing is even more important than in other species of the same botanical family (Poaceae), because maize presents low vegetative plasticity, limited capacity to compensate empty spaces through tillers, number and size of leaves (SANGOI, 2001; LEOLATO et al., 2017). In addition, maize plants have a small capacity to develop new reproductive structures such as number and size of ears, number and weight of grains in response to the reduction in plants by area (SANGOI, 2001; FROMME et al., 2019). Ensuring the uniformity sowing, crop establishment and seedling growth is the first step for obtain productive crops and determines the success or failure of the future harvest, especially in maize crops (FINCH-SAVAGE; BASSEL, 2015).

Seeds when taken to the field are subject to adverse environmental conditions, making it essential to use seeds with high physiological quality, which is directly related to the potential of germination and vigour and affected by the genotype (OLIVEIRA et al., 2013, NERLING et al., 2013, PRAZERES et al., 2016). When seeds of different genotypes are submitted to ideal conditions of temperature and humidity, the germination potential can be expressed, with the formation of normal seedlings. However, when these same cultivars are exposed under stressful conditions, the formation of normal seedlings can be impaired according to the differences in the vigour levels of the cultivars (MARCOS FILHO, 2015; FINCH-SAVAGE;BASSEL, 2015).

Researches on maize in recent years are usually specific for the relationship between physiological quality and the factors that affect it such as storage components (NERLING et al., 2018; ABREU et al, 2016; PRAZERES; COELHO, 2016), enzymes (OLIVEIRA et al., 2015; SANTOS et al., 2016; NETA et al., 2015; LOPES et al., 2017; DINIZ et al., 2018), heterosis (NERLING et al., 2013; OLIVEIRA et al., 2015; PRAZERES; COELHO, 2016; PRAZERES; COELHO, 2016; ABREU et al., 2018) and image analysis (PINTO et al., 2015; DIAS et al., 2015; CASTAN et al., 2018; MEDEIROS et al., 2018).

However, no research was found evaluating the process of dynamic reserves and seedling formation to explain the vigour of maize seeds. Researches of this nature may contribute to the understanding of the seedling formation process from seeds with different levels of initial vigour, considering that higher vigour seeds are expected to produce more vigorous seedlings (EGLI; RUCKER, 2012). Thus, the characteristics of use of seed reserves, such as the reduction of seed reserves and the mobilization for formation of new seedling tissues are important parameters of vigour evaluation (SOLTANI et al., 2006; CHENG et al., 2015).

The studies found in the literature generally evaluate seed vigour or seedling vigour separately and in that sense, have been reported in chickpea, wheat, rice, soybean and sweet corn (SOLTANI et al., 2002; SOLTANI et al., 2006; MOHAMMADI et al., 2011; CHENG et al., 2013; CHENG et al., 2015; PEREIRA et al., 2015; CHENG et al., 2018).

In this work we propose the understanding of the two concepts in an associated way through the evaluation of seedling performance parameters to help in the understanding of the behaviour of different genotypes and with that, to help the breeding programs, indicating the variable (s) that most contribute to the selection of the best cultivars for physiological quality of maize seeds.

SOLTANI et al. (2006) described that seedling growth could be measured by the weight of mobilised seed reserves (in $mg.seedlings^{-1}$) and conversion efficiency of mobilised seed reserve to seedling tissue ($mg.mg^{-1}$). Based on this principle, the study of the dynamics of seed reserves for seedling formation could be studied as an alternative method of seed vigour determination. PEREIRA et al. (2015), in a study about the dynamics of seed reserves of soybean cultivars, concluded that there is a correlation between dry seed mass, seed reserves reduction and dry seedling mass. For the results of reduction of seed reserves, conversion efficiency of reserves and dry mass of seedlings, the same authors found a negative correlation between the variables.

The selection of cultivars with greater vigour is fundamental in the management practices to obtain crops with high productivity, due to the greater capacity to overcome the adverse conditions in field conditions (MARCOS-FILHO, 2015; FINCH-SAVAGE; BASSEL, 2015) and due to stand uniformity (EGLI; RUCKER, 2012; FROMME et al., 2019). Also, the understanding of the mechanisms of how the dynamics of the reserve components during germination and seedling formation is determinant for the contribution to the advancements of seed technology (SOLTANI et al., 2006; EGLI; RUCKER, 2012).

Therefore, the objectives of this study were: (I) to verify which accelerated ageing temperature is the most effective in the separation of the vigour levels of hybrids; (II) to evaluate the dynamics of the reserves during germination and the seedling process in maize seeds with different vigour levels; (III) to perform correlations between the characteristics evaluated and (IV) to use multivariate analysis tools to identify the characteristics that contributed most to the vigour of hybrid maize seeds.

3.3 MATERIAL AND METHODS

All the experiment was developed at the Laboratory of Seed Analysis of the State University of Santa Catarina (UDESC), using the completely randomized statistical design (CRD). Seeds of seven cultivars of hybrid maize were submitted to the tests of germination rate, accelerated ageing test, moisture degree, one thousand seed weight, total seed length, shoot and root length, dry matter of seed and seedling, remaining dry matter in the endosperm, reduction of seed reserves, conversion efficiency of seed reserves and reserves mobilisation rate to the seedling.

Seven cultivars of hybrid maize from the 2016/17 crop were harvested, processed and stored in a humidity and temperature controlled chamber (45 ± 5% and 10 ± 2 °C) until the beginning of the physiological analyses. The mean samples of 1000 g of each cultivar were homogenized and divided to obtain 4 repetitions of 250 g using a sample divider (COELHO et al., 2010). To determine the germination rate, we used eight replications of 50 seeds for each hybrid, which were distributed in a germitest paper roll, moistened with distilled water in the proportion of 2.5 times the dry paper weight, according to the Rules for Seed Analysis (BRASIL, 2009). The rolls were packed in plastic bags, carried to the mangelsdorf germinator vertically and kept at 25 ± 2 ºC for 7 days. The evaluation of germination rolls was performed at 4 days (1st count) and at 7 days (2nd count). The percentage of germination is the average result of the number of normal seedlings, in percentage.

Seed vigour was determined by accelerated ageing test. The seeds were distributed in a single layer on an aluminium screen, which were placed in boxes of polystyrene crystals (gerbox) containing 40 mL of distilled water (MARCOS-FILHO, 1999). The boxes were closed and placed in accelerated ageing chamber at 43 °C for 72 hours (AOSA, 1983) and at 45 °C for 72 hours (BITTENCOURT; VIEIRA, 2006), separately. After this period, the seeds were submitted to the germination test, as previously described. Four replicates of 50 seeds were used for each hybrid and the results were expressed as percentage of normal seedlings.

The moisture degree of the seeds was determined using the oven method at 105 ± 3 °C, transferring a 4.5 ± 0.5 grams of seeds to aluminium capsules using two replications for each hybrid. After 24 hours, the capsules were weighed and the mean moisture percentage was obtained as indicated in the Rules for Seed Analysis (BRASIL, 2009).

The one thousand seed weight was performed using eight replications of 100 seeds. Each replication was weighed on analytical weighing-machine and the mean was multiplied by 10, with the final result expressed in grams as indicated in the Rules for Seed Analysis (BRASIL, 2009).

To determine the dry matter of seed, three replications of 20 seeds for each hybrid were weighed and the results were multiplied by 1000 and subtracted by the moisture degree of each sample, expressed in mg.seed^{-1} (SOLTANI et al., 2006). The same 20 seed samples obtained to determine the dry weight of seeds for each hybrid were used to total seedling length, distributed on a line drawn in the upper third of the germitest paper moistened with distilled water in the ratio 2.5 times the weight of the dry paper. The total length of 10 normal seedlings per replicate, taken at random, was measured with a digital calliper in millimetres. The evaluations were performed 120 hours (5 days) after the start of the test. The mean length of the seedlings was divided by the number of normal seedlings, with results expressed in mm.seedling^{-1}, according to the method proposed by NAKAGAWA (1999). The shoot length of 10 normal seedlings per replicate, taken at random, was measured with a digital calliper in millimetres. The evaluations were performed 120 hours (5 days) after the start of the test. The mean shoot length was divided by the number of normal seedlings, with results expressed in mm.seedling^{-1}, according to NAKAGAWA (1999). The root length of 10 normal seedlings per replicate, taken at random, was measured with a digital calliper in millimetres. The evaluations were performed 120 hours (5 days) after the start of the test. The mean root length was divided by the number of normal seedlings, with results expressed in mm.seedling^{-1}, according to NAKAGAWA (1999).

To determine the dry matter of seedling, three replications of 20 seeds were used for the seven hybrids, distributed in a line drawn in the upper third of the germitest paper, moistened with distilled water in the proportion of 2.5 times the dry paper weight and kept in germinator at 25 ± 2 °C. After 120 hours (5 days) of the start of the test, 10 normal seedlings were taken at random and the embryos (embryonic axis + scutellum structures) were separated from the endosperms, weighed and dried in an oven at 80 °C for 24 hours. The samples were weighed again and the results in mg were divided by the number of normal seedlings obtained in the rolls and the dry matter of seedlings was expressed in mg.seedling^{-1}, according to NAKAGAWA (1999) by the following expression:

$$DMSL = \frac{(Initial\ weight\ of\ the\ embryo\ -\ Final\ weight\ of\ the\ embryo)}{10} \times 1000$$

As described previously, the endosperms were separated from the embryos, weighed and dried in an oven at 80 °C for 24 hours to determine the remaining dry matter in the endosperm. The samples were weighed again and the results in mg were divided by the number of normal seedlings obtained in the rolls and the dry matter of seedlings was expressed in mg.seedling^{-1}, according to NAKAGAWA (1999).

$$RDME = \frac{(Initial\ weight\ of\ the\ endosp\ -Final\ weight\ of\ the\ endosp)}{10} \times 1000$$

To reduction of seed reserves, we subtracted the dry matter of seed (mg.seed^{-1}) by the remaining dry matter in the endosperms (mg.endosperm^{-1}), as a following expression. The results were expressed in mg.mg^{-1} (SOLTANI et al., 2006).

$$RSR = DMS\ - RDME$$

To conversion efficiency of seed reserves, we divided the dry matter of the seedlings by the reduction of seed reserves as a method to determine how much of dry matter the seed had at the beginning of the test and how much of that mass was converted to dry matter of seedling, according to SOLTANI et al. (2006). The results were expressed in mg.mg^{-1}.

$$CESR = \frac{DMSL}{RSR} \quad or \quad CESR = \frac{DMSL}{(DMS\ - RDME)}$$

Seed reserves reduction rate was also determined. This relation allows the identification of the cultivars that mobilised the most dry mass during the period of 120 hours (5 days), evaluating the real reduction of reserves, being a more reliable and comparable variable, since it is not influenced by external factors such as the dry mass of the seed. The rate was calculated by the following expression, according to SOLTANI et al., (2006):

$$SRRR = \frac{RSR}{DMS} \times 100 \quad or \quad SRRR = \frac{(DMS - RDME)}{DMS} \times 100$$

To calculate the reserves mobilisation rate to the seedling, we divided the dry matter of seedling by the dry matter of seed. The results were expressed in percentage.

$$RMRS = \frac{DMSL}{DMS} \times 100$$

Data were analysed in software R (R CORE TEAM, 2019, version 3.5.3) using scripts developed by the research group itself. The normality of the data was tested by the Shapiro-

Wilk test and the homogeneity of the variances was tested by the Levene test before analysis of variances. The test of means comparisons used was SCOTT-KNOTT (1974) at 5% ($p<0.05$) probability. The percentage data (GR and AA) were transformed using arcsin $\sqrt{x}/100$ to meet the theoretical assumptions of the F test (normal distribution of error and homogeneity of variances), although the results were presented in the original scale (%). Pearson correlations were obtained among the evaluated characteristics at 1% ($p<0.01$) and at 5% ($p<0.05$) probability. Multivariate analyses of PCA (Principal Component Analysis) and HCA (Hierarchical Cluster Analysis) were used to better visualise the discrimination of the high and low vigour groups and the variables that contributed the most to the separation of the groups.

3.4 RESULTS AND DISCUSSION

Analysis of Variances (ANOVA) showed differences among the hybrids by the F test at 5% probability ($p<0.05$) for almost all the studied characteristics (Table 2 and Table 3), except for the conversion efficiency of seed reserves (CESR).

Table 2- Averages of germination rate (GR), accelerated ageing test at 43 °C to 72 hours (AA43), accelerated ageing test at 45 °C to 72 hours (AA45), moisture degree (MD), one thousand seeds weight (OTSW), total seedling length (TSL), shoot length (SL) and root length (RL) of 7 maize hybrids and group formation by the Scott-Knott criterion.

Hybrids	**MD**	**GR**	**AA43**	**AA45**	**OTSW**	**TSL**	**SL**	**RL**
		%		**g**		**mm.seedling^{-1}**		
32R48VYHR	13	99 a[1]	96 a	76 b	293.0 c	244.2 b	76.5 a	167.7 b
30F53VYH	13	98 a	81 b	24 d	274.3 d	205.9 b	58.2 b	147.8 b
DKB230PRO3	11	97 a	95 a	93 a	259.3 e	230.7 b	61.8 b	168.9 b
30R50VYH	12	97 a	94 a	55 c	333.1 a	228.0 b	58.3 b	169.7 b
P1630H	13	97 a	88 b	40 d	272.7 d	226.3 b	61.6 b	164.7 b
P2866H	13	95 b	95 a	81 b	276.5 d	294.4 a	79.3 a	215.1 a
30F53VYHR	13	94 b	86 b	35 d	324.2 b	226.9 b	66.4 b	160.4 b
Means	13	97	91	61	294.9	236.6	66.0	170.6
C.V.	-	5.57	4.78	12.79	1.53	5.16	5.80	7.62

[1]Means followed by the same letter in each column belong to the same group according to the Scott-Knott grouping criteria at 5% probability ($p<0.05$).
Source: Elaborated by the author, 2019.

Table 3 - Averages of dry matter of seed (DMS), dry matter of seedling (DMSL), remaining dry matter in the endosperm (RDME), reduction of seed reserves (RSR), conversion efficiency of seed reserves (CESR), seed reserves reduction rate (SRRR) and reserves mobilisation rate to the seedling (RMRS) of 7 maize hybrids and group formation by the Scott-Knott criterion.

Hybrids	DMS	DMSL	RDME	RSR	CESR	SRRR	RMRS
	mg.seed^{-1}	mg.seedling^{-1}	mg.endo^{-1}	mg	mg.mg^{-1}	%	
32R48VYHR	260.3 c	95.4 a	138.7 c	121.5 a	0.79 a	46.7 a	36.6 a
30F53VYH	240.2 d	74.2 c	148.4 b	91.8 d	0.81 a	38.2 b	30.9 b
DKB230PRO3	224.5 e	81.9 b	121.3 e	103.2 c	0.79 a	46.0 a	36.5 a
30R50VYH	286.2 a	83.9 b	184.8 a	101.3 c	0.83 a	35.4 b	29.3 b
P1630H	237.4 d	65.1 d	151.8 b	85.5 d	0.76 a	36.0 b	27.4 b
P2866H	240.3 d	84.4 b	130.9 d	109.4 b	0.78 a	45.5 a	35.2 a
30F53VYHR	277.8 b	88.6 b	177.2 a	100.6 c	0.88 a	36.2 b	31.9 b
Means	252.4	81.9	150.4	101.9	0.81	40.6	32.5
C.V.	1.34	5.21	3.56	5.72	5.48	5.19	5.66

[1]Means followed by the same letter in each column belong to the same group according to the Scott-Knott grouping criteria at 5% probability ($p<0.05$).
Source: Elaborated by the author, 2019.

For the moisture degree (MD), values from 11% to 13% were observed, indicating that all hybrids presented similar conditions of initial moisture for the experiment. Despite the difference between the moisture levels of the cultivars, this was not considered compromising the results of the tests, since it is recommended that the difference between the cultivars does not exceed 2% to avoid increasing the deterioration intensity and to reduce the chemical, physiological and sanitary changes in seeds (MARCOS-FILHO, 1999; BARROZO et al., 2014; DIAS et al., 2015). The moisture degree determination was used only as a method to control the initial conditions of the seeds used in the experiment.

Apart from that, all hybrids presented germination rate (GR) higher than 90% (from 94% to 99%), that is, despite the existence of a statistically significant difference between them, all presented a high germination potential (Table 2). This potential is important in establishing crops because it determines the plant stand and the final productive potential of the crop until the seedling ceases to depend on seed reserves and becomes autotrophic (BEWLEY et al., 2013; FINCH-SAVAGE; BASSEL, 2015).

It was observed with our results that there were differences in the physiological potential (germination and vigour) among the hybrid maize genotypes used in the experiment (Table 2). In study carried out by NERLING et al. (2013) with crosses among maize varieties, it was observed that there was genotype effect on germination and seed vigour, where these authors verified the importance of analysing the physiological quality to define the potential of the genotype and its crosses due to the presence of heterosis.

When the seeds were submitted to stress by accelerated ageing, it was observed that at 43 °C (AA43) was possible to separate the hybrids in two levels of vigour by the Scott-Knott test ($p<0.05$), with values ranging from 81% to 96% (Table 2). Four of the seven hybrids evaluated showed higher vigour (32R48VYHR, DKB230PRO3, 30R50VYH and P2866H), while the other three hybrids presented lower vigour (30F53VYH, P1630H and 30F53VYHR).

However, for studies on the understanding of the expression of seed vigour, it is important to use cultivars that are contrasting in this characteristic, but which present high germination potential (DIAS et al, 2015). Thus, it was necessary to use another stress condition in order to establish greater contrasts among the hybrids and to permit the selection of the most distinct cultivars possible. In the evaluation of the physiological quality of the seeds, it is important to use vigour determination methods capable of identifying the differences between the individuals, especially when the germination power between them is similar (MARCOS-FILHO, 1994).

When the seeds were submitted to stress by accelerated ageing at 45 °C (AA45), it was possible to separate the hybrids in more efficient and contrasting levels of vigour by the Scott-Knott test ($p<0.05$), forming four groups, varying from 24% to 93% of vigour (Table 2). The most vigorous hybrid by this test (AA45) was the DKB230PRO3, with 93% and the less vigorous were the 30F53VYH, P1630H and 30F53VYHR, with values of 24%, 40% and 35% of vigour, respectively. These same hybrids were the ones that showed low vigour when the temperature was less severe, indicating the higher sensitivity of them to this stress.

For the condition of our experiment, AA45 was the most effective in the sense of separation of vigour levels and it was reported by other authors. BITTENCOURT; VIEIRA (2006) tested the combinations of two temperatures (42 and 45 °C) and two exposure periods (72 and 96 hours) to ageing stress on maize seeds and concluded from their results that the combination of 45 °C for 72 hours was the one that promoted segregation more advantageous related to vigour levels, especially when the lots have similar germination potential, such as those that occurred in this present study. NERLING et al., (2015) and PRAZERES; COELHO (2016) defined accelerated ageing stress at 45 °C for 72 hours as the variable that contributed the most to define the physiological potential of maize varieties and hybrids.

In relation to the one thousand seeds weight (OTSW), significant differences were observed between the cultivars, with values varying from 259.3 g to 333.1 g (Table 2). It was observed that the hybrid with the highest vigour (DKB230PRO3) was the one with the lowest OTSW, indicating absence or relation between seed size and vigour. Moreover, for the results of dry matter of seed (DMS), we observed the same separation of means by Scott-Knott test

found for OTSW, where the hybrid of highest vigour (DKB230PRO3) was the one that presented the lowest DMS value (224.5 mg.seed^{-1}) (Table 3).

The influence of maize seed size on vigour has not yet been fully elucidated by scientific research. In this study, the results showed that vigour did not depend on the size of the seed evaluated by the weight of one thousand seeds and the dry mass of seeds. These same results were reported by MOLATUDI; MARIGA (2009), who tested sowing of large and small seeds at different depths and concluded that seed size has no effect on seedling emergence and vigour, although greater depths significantly affect these parameters. In addition, SULEWSKA et al. (2014), who evaluated the growth of seedling, alpha-amylase enzyme activity and yield of maize seeds of different sizes (different one thousand seeds weight) for three years. These authors concluded that smaller seeds present higher germination, higher activity of the alpha-amylase enzyme, lower seedling growth rate and higher productivity. On the other hand, other authors argue that when maize seed size is larger, seedling vigour is increased, especially when sowing depth is higher (EL-ABADY, 2015).

The fact that seeds of smaller size present high vigour can be explained by the higher seed reserves reduction rate (SRRR) that these hybrids presented, where there was a greater reduction of seed reserves (RSR), less remaining dry matter in the endosperm (RDME) and, consequently, higher availability of nutrients to be mobilised for the growth and development of seedlings. This mobilisation was evaluated by the reserves mobilisation rate to the seedling (RMRS), which represents the ability of the cultivar to remove nutrients from the storage tissue and convert them to seedlings, although the conversion efficiency of seed reserves (CESR) was similar among hybrids.

The seed reserves reduction rate (SRRR) is the ratio between the reduction of seed reserves (RSR) and the dry matter of seed (DMS), in other words, is the amount of mass that has been removed from the reserve tissue for maintenance of metabolism and for the development of the seedling. On the other hand, the reserves mobilisation rate to the seedling (RMRS) represents how much was actually used for the growth and development of the embryonic axis during germination, which means the ability that a hybrid has possessed in using what has been removed from the seed to form a normal seedling during the evaluation period (after 120 hours of sowing).

We calculated the seed reserves reduction rate (SRRR) and the reserves mobilisation rate to the seedling (RMRS) and we observed that the hybrids with higher vigour (DKB230PRO3, 32R48VYHR and P2866H) were those that presented the highest rates, with values of 46.0, 46.7 and 45.5% of SRRR, respectively. The values of RMRS were 36.5, 36.6

and 35.2%, respectively. That is, they have a greater ability to use the endosperm reserves to form the seedling, although the conversion efficiency of seed reserves of the hybrids has been similar. With these analyses used in the experiment, we elucidate what occurs physiologically with seed reserves during germination and seedling formation in hybrids with different vigour levels.

The highest RMRS were found in the most vigorous hybrids. This result was found by EHRHARDT-BROCARDO; COELHO (2016) in common bean seeds (*Phaseolus vulgaris*). These authors evaluated the seedling performance test and its relation with the physiological quality of common bean seeds and found that seeds with greater potential for converting cotyledon reserves to seedling formation were the ones with the best vigour by accelerated ageing and longer seedling length, corroborating with the results found in this present study. Seeds that present a lower rate of mobilisation of reserves for the formation of seedlings gave rise to smaller seedlings (EHRHARDT-BROCARDO; COELHO, 2016), according to our results with maize seeds.

According to the results obtained in this study, 23 significant correlations between the variables evaluated were found at 1% probability and 41 significant correlations at the 5% level (Table 4).

There were significant correlations between initial seed vigour by accelerated ageing (AA45) and seedling performance variables (TSL, SL, RL, RDME, RSR, SRRR and RMRS). When vigour by accelerated aging was higher (AA45), total seedling length was higher (r=+0.60), as was shoot and root length (r=+0.48, r=+0.56).

On the other hand, the remaining dry matter in the endosperm was lower (r=-0.61), because the reduction of seed reserves was higher (r=+0.60). Thus, the seed reserves reduction rate was improved (r=+0.77) and the reserves mobilisation rate to the seedling (r=+0.66) was higher in these cultivars (32R48VYHR, DKB230PRO3 and P2866H).

Table 4- Results of the Pearson correlation between the 14 variables evaluated based on the mean of the hybrids. **, *: Significant at 1% (p<0.01) and 5% (p<0.05) probability by the t test. GR – Germination Rate; AA43 – Accelerated Ageing test at 43 °C to 72 hours; AA45 – Accelerated Ageing test at 45 °C to 72 hours; OTSW – One Thousand Seeds Weight; TSL – Total Seedling Length; SL – Shoot Length; RL – Root Length; DMS – Dry Matter of Seed; DMSL – Dry Matter of Seedling; RDWE – Remaining Dry Matter in the Endosperm; RSR – Reduction of Seed Reserves; CESR – Conversion Efficiency of Seed Reserves; SRRR – Seed Reserves Reduction Rate; RMRS – Reserves Mobilisation Rate to the Seedling.

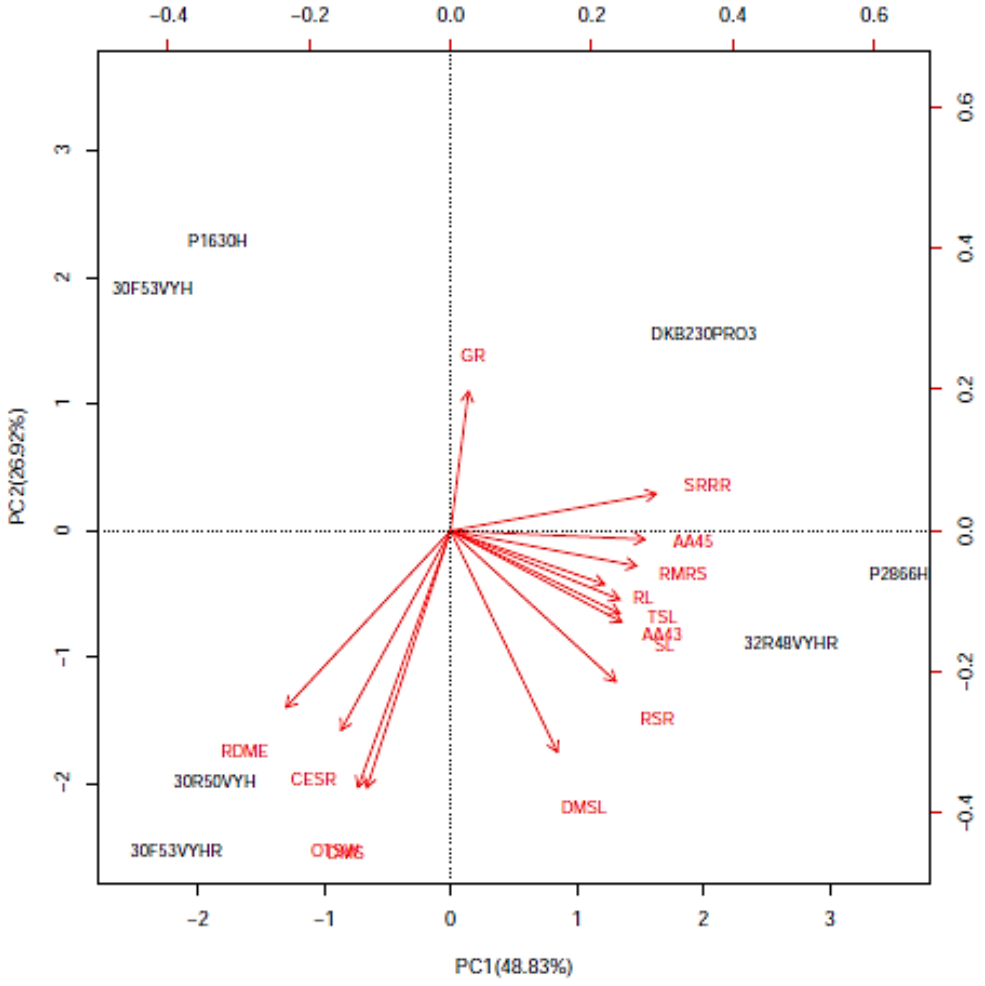
	GR	**AA43**	**AA45**	**OTSW**	**TSL**	**SL**	**RL**	**DMS**	**DMSL**	**RDME**	**RSR**	**CESR**	**SRRR**	**RMRS**
GR	-	0.02	0.09	-0.29	-0.25	-0.21	-0.23	-0.22	-0.14	-0.20	-0.02	-0.24	0.10	-0.01
AA43	-	-	0.70**	0.01	0.44*	0.46*	0.37	0.01	0.43	-0.26	0.52*	-0.15	0.47*	0.46*
AA45	-	-	-	-0.30	0.60**	0.48*	0.56**	-0.30	0.38	-0.61**	0.60**	-0.35	0.77**	0.66**
OTSW	-	-	-	-	-0.12	-0.08	-0.12	0.98**	0.40	0.88**	0.13	0.51*	-0.51*	-0.30
TSL	-	-	-	-	-	0.73**	0.96**	-0.13	0.33	-0.40	0.51*	-0.25	0.55*	0.46*
SL	-	-	-	-	-	-	0.52*	-0.04	0.58**	-0.40	0.66**	-0.07	0.62**	0.64**
RL	-	-	-	-	-	-	-	-0.15	0.20	-0.34	0.37	-0.29	0.44*	0.32
DMS	-	-	-	-	-	-	-	-	0.45*	0.85**	0.22	0.45*	-0.45*	-0.27
DMSL	-	-	-	-	-	-	-	-	-	-0.02	0.85**	0.37	0.50*	0.74**
RDME	-	-	-	-	-	-	-	-	-	-	-0.32	0.52*	-0.85**	-0.66**
RSR	-	-	-	-	-	-	-	-	-	-	-	-0.17	0.77**	0.74**
CESR	-	-	-	-	-	-	-	-	-	-	-	-	-0.42	0.08
SRRR	-	-	-	-	-	-	-	-	-	-	-	-	-	0.87**
RMRS	-	-	-	-	-	-	-	-	-	-	-	-	-	-

**, *Significant at 1% (p <0.01) and 5% (p <0.05) of probability by the t test.
Source: Elaborated by the author, 2019.

In this present study, we evaluated the TSL, SL and RL variables and the results showed that there was a significant difference in total seedling length (TSL), shoot length (SL) and root length (RL) by comparison among the hybrids, indicating the difference in vigour between them. There was a high correlation between the vigour test by accelerated ageing (AA45) and the seedling performance test for total seedling length (TSL), shoot length (SL) and root length (RL). SENA et al. (2017), evaluating the sensitivity of different seedling performance tests for vigour evaluation in 20 maize seed lots, found that shoot length and root length were the most sensitive variables for vigour classification at different levels.

We applied two multivariate analysis tools (PCA and HCA) in a complementary way to the Pearson correlation analysis. For the principal component analysis (PCA), the total variance explained by the two main components was 75.75%, with 48.73% of the variance explained by PC1 and 26.92% explained by PC2 (Figure 2).

Figure 2- Scores of the first and second Principal Components (PC1 and PC2) for seedling performance variables in seven cultivars of hybrid maize with different levels of vigour.

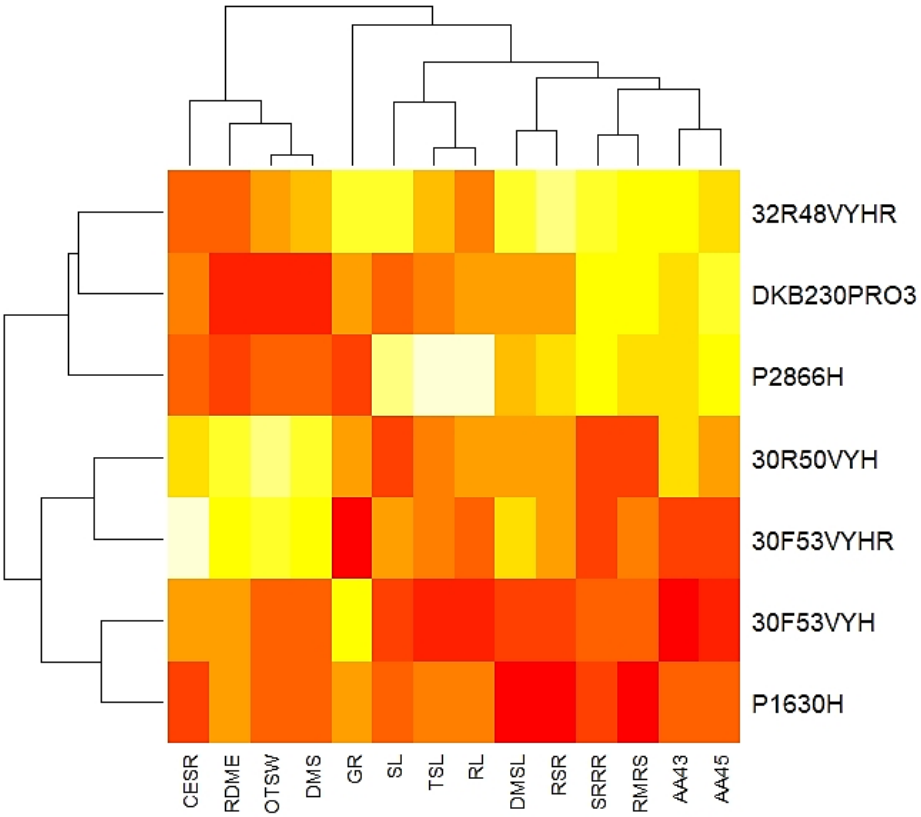


Source: Elaborated by the author, 2019.

The loading values showed that the hybrid classified as highest vigour was grouped in PC1+/PC2+ by the germination rate (GR) and seed reserves reduction rate (SRRR) variables. The others high vigour hybrids 32R48VYHR and P2866H were grouped in PC1+/PC2- by the variables of accelerated ageing (AA43 and AA45), reserves mobilisation rate to the seedling (RMRS), root length (RL), shoot length (SL) and total seedling length (TSL), reduction of seed reserves (RSR) and dry matter of seedling (DMS). However, the hybrids classified as low vigour 30R50VYH and 30F53VYHR were grouped in PC1-/PC2- by the variables of remaining dry matter in the endosperm (RDME), conversion efficiency of seed reserves (CESR), one thousand seeds weight (OTSW) and dry matter of seed (DMS). Finally, the other two low-vigour hybrids P1630H and 30F53VYH were clustered into PC1- / PC2 +.

The heat map of Hierarchical Cluster Analysis (HCA) shows that there were two main groups, where the highest vigour cultivars (32R48VYHR, DKB230PRO3 and P2866H) were in the same group, while the lower vigour cultivars (30R50VYH, 30F53VYHR, 30F53VYH and P1630H) were in another group (Figure 3). It was observed that the germination rate (GR) variable was grouped separately from the other variables and in the intermediate area of the cluster, since it was important for all the hybrids.

Figure 3 - Cluster heat map for the dynamics of seedling formation and physiological quality of seven maize seeds.



Source: Elaborated by the author, 2019.

It was observed that the cultivars classified as higher vigour were grouped by PCA and HCA (Figure 2 and Figure 3) in a different group of the cultivars classified as lower vigour, where the variables that contributed the most to this separation were the same ones found in the Pearson correlation positively correlated with vigour by accelerated ageing (RMRS, SRRR, DMSL, RSR, RL, TSL and SL). On the other hand, for the hybrids of lower vigour, the variables that contributed the most to the separation were those that did not correlate significantly with the vigour (OTSW, DMS, CESR), or that there was a negative correlation (RDME). Thus, the parameters of stored reserves dynamics and seedling formation evaluated in this study explained the vigour in hybrid maize seeds and can be used as a method of evaluation of the physiological quality.

The results of this study suggest that more detailed research regarding the study of enzymatic activity and/or reserve components involving the seed reserve reduction rate (SRRR) and the reserves mobilisation rate to the seedling (RMRS) should be done, mainly using the two most contrasting cultivars for the level of vigour by accelerated ageing (DKB230PRO3 and 30F53VYH). This efforts should be done mainly to understand and elucidate metabolically why the differences on vigour exist, identifying the mechanism that reflects the expression of vigour in seeds of hybrid maize, since the physiological changes occurred in these cultivars were presented and discussed in this article.

3.5 CONCLUSION

There is genetic variation for the initial seed vigour and vigour of the seedlings. The dynamics of seed reserves and seedling formation depends on the genotype and the initial vigour evaluated by accelerated ageing.

Accelerated ageing at 45 °C for 72 hours (AA45) is the most efficient combination to segregate vigour levels of hybrid maize seeds.

Higher vigour hybrids by AA45 present higher seed reserves reduction rate (SRRR) and higher reserves mobilisation to the seedling (RMRS), producing seedling with higher dry matter of seedling (DMSL), higher total seed length (TSL), shoot length (SL) and root length (RL), regardless of seed size.

PCA and HCA were efficient tools to identify that the variables AA45, SRRR and RMRS were the most important for the differentiation of the vigour of the hybrids.

Our results prompt us to conclude that these two rates may explain the vigour in hybrid maize seeds and can be used in breeding programs aimed at the selection of cultivars with high physiological quality.

4 CHAPTER 2 – PHYSIOLOGICAL AND BIOCHEMICAL PROFILING OF TWO CONTRASTING MAIZE HYBRIDS SUBMITTED TO ACCELERATED AGEING TEST

4.1 ABSTRACT

Finding the understanding of what occurs in the metabolism of seeds during the deterioration process allows the intervention in biochemical components and the prevention of the reduction of seed quality. The main objectives were: (i) to determine if the physiological and biochemical changes caused by accelerated ageing explain the high and low vigour of maize seeds; (ii) to verify if the main biochemical changes in response to stress occur in the endosperm or embryo (iii) using statistical tools (Pearson Correlation, PCA, HCA and PLS-R) to determine the variable that most influenced the vigour response. A completely randomized design (CRD) was used in a 2x5 factorial arrangement, with 2 contrasting hybrids in the level of vigour (H1 - high vigour and H2 - low vigour) and 5 accelerated ageing stresses at 45 °C (0, 12, 24, 48 and 72 hours of stress). Physiological (normal seedlings, abnormal seedlings, unviable seeds, electrical conductivity, moisture degree) and biochemical analysis (total soluble sugar, starch, α-amylase, soluble protein, electrophoresis gel of protein, enzyme activity of SOD and CAT, hydrogen peroxide and malondialdehyde) were performed in the embryo and endosperm. The higher tolerance of hybrid maize seeds to accelerated ageing stress is dependent on the higher total soluble sugars, starch and total soluble protein content of the embryo and endosperm. On the other hand, the higher sensitivity to stress is associated with seeds with higher membrane instability and higher lipid peroxidation in the embryo and endosperm of hybrid maize seeds. Evaluating soluble sugars, starch and total soluble protein content components brings early information on the physiological quality of the seeds and can be used to select seeds with better physiological quality. The behaviour of these components during accelerated ageing explains the changes in the vigour of hybrid maize seeds.

4.2 INTRODUCTION

The world population growth has been growing substantially, requiring development in production and quality of food (HAMPTON et al., 2016). According to the United States Department of Agriculture (USDA, 2019), maize (*Zea mays* L.) is considered the most cultivated cereal in the world. Thus, studies that contribute to improve the management and productivity of this crop have gained importance (PECHANOVA; PECHAN, 2017).

Maize grains or seeds are considered botanically a dry fruit, called caryopsis, which is formed basically of four structures: endosperm, embryo, pericarp and pedicel (BEWLEY et al., 2013). The endosperm constitutes the major part of the maize seed, being mainly formed by starch (86.4 %) and protein (9.4 %) (CARVALHO; NAKAGAWA, 2012; TOSELLO, 1987). The embryo, which is formed by the union of the embryonic axis and the single cotyledon, called scutellum (BEWLEY et al., 2013), is composed mainly of lipids (34.5 %), proteins (18.8 %) and sugars (10.8 %) (CARVALHO; NAKAGAWA, 2012; TOSELLO, 1987).

Seed germination process is essential for the development of a new plant and, ultimately, to achieve high crop yields, especially in maize, which is a specie dependent on the uniformity and initial establishment (FINCH-SAVAGE; BASSEL, 2015; MARCOS-FILHO, 2015; HAMPTON et al., 2016). The manifestation of the phenotype (i.e., normal and vigorous seedlings) can be attributed to the chemical composition of the seed (SANTOS et al., 2017), because seed reserves are essential as a source of energy required to maintain the physiological and biochemical mechanisms during germination (COELHO; BENEDITO, 2008; BEWLEY et al., 2013; YU et al., 2014).

However, when seeds are sown in the field, they are predisposed to biotic and abiotic conditions that are unfavourable for their development. Thus, a complex feature, called seed vigour, becomes essential for seed germination and establishment of seedlings under these conditions (CORBINEAU, 2012; RAJJOU et al., 2012; HAN et al., 2014; MARCOS FILHO, 2015). The study of the changes of these components during stress may bring relevant information about the seed vigour. In order to improve seed vigour, it is necessary to understand the biochemical and physiological mechanisms associated with it (SUN et al., 2007). Understanding this complex feature is a research challenge and remains unknown, especially in maize seeds.

One of the main forms of energy supply to the embryo is the glycolysis of phosphorylated sugars (HAN et al., 2017). These soluble sugars may be derived from the gluconeogenesis or the hydrolysis of polysaccharides, such as starch, by the action of amylases

(HAN et al., 2017). As maize seeds are generally composed mainly of starch, the activity of the α-amylase enzyme and changes in the content of starch and soluble sugars during stress conditions may be an important indicator of seed vigour (ZHANG et al., 2007; PRAZERES; COELHO, 2016). The association of maize seed vigour with amylase enzyme activity has been well established by the research, where the highest vigour is associated with the higher activity of this enzyme (OLIVEIRA et al., 2013; OLIVEIRA et al., 2015; SANTOS et al., 2015; NERLING et al., 2018). However, the evaluation of the enzymatic activity during the ageing process has not yet been elucidated.

There are many tests to evaluate seed vigour. One of the most widely used tests for several species, including maize, is the accelerated ageing test, which involves subjecting seeds to high temperature (> 40 °C) and saturated relative humidity conditions for a specific period (DELOUCHE; BASKIN, 1973; MARCOS-FILHO, 1999; MARCOS-FILHO, 2015). High-vigour seeds can produce normal seedlings after ageing while low-vigour seeds produce abnormal seedlings or die (HARMAN; MATTICK, 1976; HAN et al., 2014; MARCOS-FILHO, 2015).

Many authors have reported that one of the main causes of ageing deterioration is the chemical oxidation associated with the presence of reactive oxygen species, such as hydrogen peroxide, superoxide and hydroxyl radicals, and the singlet oxygen, which are toxic compounds to the cells formed in stress situations (BAILLY, 2004; KUMAR et al., 2015). In this sense, antioxidant mechanisms, such as the presence of enzymes (e.g. superoxide dismutase, catalase, amongst others) were studied and associated with higher vigour seeds, conferring greater tolerance to stress (ABREU et al., 2014; SANTOS et al., 2015; BALDONI et al., 2019).

The leakage of solutes (mainly ions, sugars and proteins) from the interior of the cells is also associated with the presence of free radicals, through the lipid peroxidation of membranes, increasing the ageing process and decreasing vigour (BEWLEY et al., 2013; MARCOS-FILHO, 2015). In addition, other problems of lipid peroxidation and protein denaturation caused by accelerated ageing are the impairment of cellular compartmentalization, coalescence of mitochondrial membranes, which can damage critical processes such as respiration, electron transport and ATP synthesis (BEWLEY et al., 2013; RATAJCZAK et al., 2019). Transcription of genes can also be compromised in seeds in the process of deterioration, since the activation of DNA repair mechanisms is dependent on the presence of water in the seed (BEWLEY et al., 2013; WU et al., 2017; WATERWORTH et al., 2019).

Although many of the causes of seed deterioration are already well established by the research, few studies have been published on the use of the accelerated ageing test to clarify the

mechanisms associated with the vigour characteristics (HAN et al., 2014, WANG et al., 2016, HAN et al., 2018). Physiological quality has not been a feature considered in traditional breeding or genetic engineering programs. However, some authors have already proven that the germination potential and vigour of maize seeds can be improved during the development of new cultivars (SANTOS et al.; 2012; NERLING et al., 2013; OLIVEIRA et al., 2013; PRAZERES; COELHO, 2016; PRAZERES; COELHO, 2016, SANTOS et al., 2017). In addition, the results found in seed research using hybrid maize as study material can be applied to other crops, especially other cereals (PECHANOVA; PECHAN, 2017).

In the present study, the first monitoring of physiological and biochemical changes during the accelerated ageing of high and low vigour hybrid maize seeds was developed. The main objectives were: (i) to determine if the physiological and biochemical changes caused by accelerated ageing explain the high and low vigour of maize seeds; (ii) to verify if the main biochemical changes in response to stress occur in the endosperm or embryo (iii) using statistical tools (Pearson Correlation, PCA, HCA and PLS-R) to determine the variable that most influenced the vigour response.

4.3 MATERIAL AND METHODS

The experiment was carried out in the Laboratory Seed Analysis of the University of Santa Catarina State. A completely randomized design (CRD) was used in a 2x5 factorial arrangement, with 2 contrasting hybrids in the level of vigour (H1 – high vigour and H2 – low vigour) and 5 accelerated ageing stresses at 45 °C (0, 12, 24, 48 and 72 hours of stress). Analyses of normal seedlings, abnormal seedlings, unviable seeds, electrical conductivity were performed in entire seeds, while analyses of moisture degree, total soluble sugar, starch, α-amylase activity, soluble protein content, electrophoresis gel of protein, SOD and CAT activity, hydrogen peroxide and MDA content were performed in the embryo and endosperm, separately.

Two hybrid maize cultivars were previously selected in Chapter 1 in relation to the vigour level by accelerated ageing at 45 °C/72 hours (four biological replicates of 50 seeds) and germination rate (eight biological replicates of 50 seeds) and were used as experimental material. The hybrids DKB230PRO3 (97% of germination rate and 93% of vigour) and 30F53VYH (98% of germination rate and 24% of vigour) were selected.

In order to evaluate normal seedlings, abnormal seedlings and unviable seeds in response to stress, the seeds were submitted to accelerated ageing during periods of 12, 24, 48

and 72 hours. Non-stressed seeds (0 hours) were used as control. The seeds were distributed in a single layer on aluminium screens, placed in gerbox boxes containing 40 mL of distilled water inside and kept at 45 °C for periods of 12, 24, 48 and 72 hours in an accelerated ageing chamber with saturated relative humidity. After each period, four replicates of 50 seeds per hybrid were distributed in rolls of germitest paper, moistened with distilled water in the proportion of 2.5 times the weight of the dry paper, placed in plastic bags and kept in germinator at 25 °C, according to the germination rate methodology proposed by BRASIL (2009). Non-stressed seeds (T0 - 0 hours of stress) were also submitted to the test. The number of normal seedlings counts was performed on the 4^{th} and 7^{th} days after the start of the test. The number of abnormal seedlings and unviable seeds were counted after 7 days. The results were expressed as percentages of normal seedlings, abnormal seedlings and unviable seeds.

To determine the electrical conductivity of the seeds in response to stress, after each accelerated ageing period at 45 °C (12, 24, 48 and 72 hours), in addition to the seeds without stress (0 hours), three replicates of 50 seeds for each stress times were weighed and placed in plastic cups containing 75 mL of distilled water (VIEIRA; KRZYZANOWSKI, 1999; MIGUEL and MARCOS-FILHO, 2002). The plastic cups were kept in germinator at 25 °C for 24 hours and the electrical conductivity readings of the seeds soaking solutions and distilled water were performed by a benchtop conductivity meter. The electrical conductivity values of the soaking solutions were subtracted by the electrical conductivity value of the water and divided by the initial weight of the 50 seeds. The mean values of electrical conductivity were expressed in $\mu S.cm^{-1}.g^{-1}$ of seed.

To determine the moisture degree of embryo and endosperm during the stress (0, 12, 24, 48 and 72 hours), the seeds had the embryo (scutellum and embryonic axis) excised from the endosperm to determine the moisture content of the structures, separately. Two replicates of 4.5 g ± 0.5 g of ground embryo and ground endosperm were used per time, placed in aluminium capsules, weighed, kept in an oven at 105 °C ± 3 °C for 24 hours and weighed again after this period. The results were expressed in percentage, according to the Rules for Seed Analysis (BRASIL, 2009).

With the purpose of collecting the samples for biochemical analysis, eight replicates of 100 seeds were used, distributed in aluminium screens and placed in gerbox boxes containing 40 mL of distilled water. The boxes were kept in an accelerated ageing chamber and after each stress period (0, 12, 24, 48 and 72 hours) at 45 °C and saturated relative humidity, the boxes of high-vigour and low-vigour seeds were removed from the chamber. The replicates were all homogenised to obtain the pool of samples to be used in the biochemical analysis. All seeds

had the embryo (scutellum and embryonic axis) separated from the endosperm, frozen with liquid nitrogen and ground to obtain the flour. The 20 samples (2 hybrids – high and low-vigour; 5 stress times – 0, 12, 24, 48, 72 hours; 2 seed structures – embryo and endosperm) were kept in ultra-freezer at -80 °C until the beginning of the analysis.

For the extraction of total soluble sugars, three replicates of 125 mg of ground embryo and ground endosperm for each stress period (0, 12, 24, 48 and 72 hours) were oven dried at 60 °C for 48 hours. The samples were placed in falcon tubes, homogenised in 12.5 mL of ethyl alcohol 80 % (v/v) and kept in a water bath at 60 °C for 15 minutes. After this step, the samples were centrifuged at 3000 rpm for 7 minutes. The supernatant was stored and in the precipitate was added 15 mL of 80 % ethyl alcohol, kept in a water bath at 60 minutes for 15 minutes and centrifuged at 3000 rpm for 7 minutes. The supernatants from the two centrifugations were homogenised and the precipitate was separated for further extraction of the starch. Aliquots of 100 µL of the embryo extracts were diluted in 900 µL of distilled water. For the reading of the samples, 20 µL of the extracts were added to the test tubes with 980 µL of distilled water and 2 mL of anthrone reagent (0.04 g anthrone, 1 mL distilled water, 20 mL sulphuric acid) prepared at the time of the use. The samples were then homogenised using a vortex mixer and kept in a water bath at 96 °C for 3 minutes. After this period, the tubes were immediately cooled for 5 minutes and the absorbance readings were performed in a spectrophotometer at 620 nm using glass cuvettes. The standard curve for the soluble sugar was obtained through a glucose solution at concentrations of: 0; 0.1; 0.2; 0.4; 0.6; 0.8 and 1.0 $\mu g.mL^{-1}$. The results were expressed as mg soluble $sugar.g^{-1}$ dry mass, according to the method proposed by CLEGG (1956).

The starch content was determined in the embryo and the endosperm of the seeds, using three replicates for each hybrid at each stress time. For extraction, 10 mL of sulphuric acid 0.2 N was added to the remaining residue from the extraction samples of the total soluble sugars. The tubes were sealed, shaken and kept in a water bath at 100 °C for 2 hours. For quantification, the anthrone method proposed by CLEGG (1956) was used. Aliquots of 10 µL of the embryo and endosperms extracts were diluted in 990 µL of distilled water. For the absorbance reading of the samples in the spectrophotometer, the endosperm samples were again diluted in centrifuge microtubes (eppendorfs), using 400 µL of extract and 600 µL of distilled water. After this step, 1 mL of diluted sample and 3 mL of anthrone reagent (0.04 g of anthrone, 1 mL of distilled water, 20 mL of sulphuric acid) prepared at the time of use were added to the test tubes. The samples were homogenised using a vortex mixer and taken to the water bath at 96 °C for 3 minutes. After this period, the tubes were immediately cooled for 5 minutes and the readings were performed in a spectrophotometer at 620 nm using glass cuvettes. The standard curve for

the starch was obtained through a glucose solution at concentrations of: 0; 0.1; 0.2; 0.4; 0.6; 0.8 and 1.0 μg.mL^{-1}. The results were corrected by the dilution factors of the samples, multiplied by 0.9 (correction factor of glucose in starch) according to MCCREADY et al. (1950) and expressed as a percentage of starch (%).

The activity of α-amylase enzyme was determined by the method proposed by GUGLIEMINETTI et al. (1995). For extraction, three replicates of 500 mg of ground embryo and ground endosperm were macerated in a mortar on ice using 10 mL of Tris-HCl buffer solution 0.1 mol.L^{-1} pH 7.0 containing sodium chloride (NaCl) 0.1 mol.L^{-1} and calcium chloride ($CaCl_2$) 10 mmol.L^{-1}. The homogenate was transferred to falcon tubes and centrifuged at 8000 rpm for 10 minutes at 4 °C. For quantification, 0.5 mL of extract was homogenised with 0.5 mL of 2.5% starch solution and 0.5 mL of buffer solution pH 5.2 containing sodium acetate ($C_2H_3NaO_2$) 50 mmol.L^{-1} and $CaCl_2$ 10 mmol.L^{-1}. The samples were kept in a water bath at 70 °C for 15 minutes. After this period, 1 mL of DNS reagent was added to the samples and incubated at 100 °C for 5 minutes. The DNS reagent was composed by 5 g of 3,5-dinitrosalicylic acid diluted in 100 mL of sodium hydroxide 2 mol.L^{-1} and 150 g of sodium and potassium double tartrate diluted in 250 mL of distilled water, which were homogeneised and the final volume was completed to 500 mL with distilled water. After cooling, 7.5 mL of distilled water was added to each test tube. The absorbance readings were made in a spectrophotometer at 540 nm using quartz cuvettes. The standard glucose curve was used at concentrations of: 0; 0.05; 0.1; 0.2; 0.4; 0.8; 1.60 and 2.00 mg.mL^{-1}. The results of the analysis were expressed in mmol of reduced sugars.g^{-1}.min^{-1}. However, the data were transformed to enzyme activity, calculated as 1 unit of activity (U) equivalent to 1 μmol of sugars produced in 1 minute under the assay conditions. Results of enzyme activity were expressed as U of enzyme.kg of seed^{-1}.

To determine the total soluble protein, three replicates of 250 mg of ground embryo and ground endosperm were used for each stress time according to the methodology proposed by AZEVEDO et al. (1998). The samples were homogenised with 2.5 mL of potassium phosphate buffer 0.1 M pH 7.5 containing ethylenediamine tetraacetic acid (EDTA) 1 mM, dithiothreitol (DTT) 3 mM and polyvinyl polypyrrolidone (PVPP) 4% (w/v) and centrifuged at 8.000 rpm for 30 minutes at 4 °C. The same extract was used for the analysis of the protein profile by the electrophoresis gel, enzymatic analysis of superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) that will be described later. Quantification was performed by the method proposed by BRADFORD (1976). Embryo samples were diluted 20 times before the quantification reaction. Aliquots of 20 μL were homogenised in centrifuge microtubes (eppendorfs) with 200 μL of Bradford reagent and 800 μL of distilled water. The readings were performed in a

spectrophotometer at 595 nm using plastic cuvettes. The standard curve was made with bovine serum albumin (BSA) fraction V at concentrations of 0.03; 0.05; 0.1; 0.2; 0.3 and 0.4 $mg.mL^{-1}$. The results were expressed in $mg.g^{-1}$ of fresh weight.

The protein profile was obtained by the polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE). The embryo and endosperm gels were made in duplicate using independent soluble protein extracts for each replicate. The resolving and stacking gel were prepared at the concentration of 12% and 4%, respectively. Soluble protein extracts were used, taking 15 μg of protein for the embryo samples and 5 μg of protein for the endosperm samples, prepared in a ratio of 1:1 using sample buffer containing distilled water, tris-(hydroximethyl)-aminomethane pH 6.7, glycerol, sodium dodecyl sulphate (SDS) 10%, bromophenol blue 0.5% and β-mercaptoethanol, according to the method proposed by LAEMMLI (1970). The samples were boiled for 5 minutes at 96 °C in a water bath and after that step, they were centrifuged and immediately applied to the stacking gel. In addition, 10 μL of protein molecular weight marker (Precision Plus Protein™ - Bio-Rad, CA, USA) was applied to the gels. All runs of the gels occurred for 2 hours at a constant current of 140 v in a mini gel system. After the run, the gels were washed with distilled water and stained in solution containing Comassie Blue R-250, methyl alcohol, glacial acetic acid and distilled water under constant gentle stirring for 3 hours. The gels were then decolourised in methyl alcohol, glacial acetic acid and distilled water for 24 hours at room temperature. Afterwards, they were washed with distilled water and submitted to the analysis of the differences in the intensity of bands between the embryos and endosperms of the high and low vigour seeds by the software GEL ANALYZER (2010), using the automatic band detection tool.

The activity of the superoxide dismutase enzyme (SOD) was determined by the method proposed by SUN et al. (1988). The soluble protein extract was used for the determination of the activity of the SOD enzyme. For each sample, three test tubes (sample, sample blank and solution blank) were covered with aluminium foil prior to the start of the quantification reaction. In the sample tube, 2 mL of sodium phosphate buffer 0.1 $mol.L^{-1}$ pH 7.8, 50 μL of the protein extract, 250 μL of nitroblue tetrazolium chloride (NBT), 200 μL of ethylenediamine tetraacetic acid (EDTA), 250 μL of methionine and 250 μL of riboflavin were added, totalling 3 mL the final reaction volume. All reagents were prepared at the time of the use. For the sample blank tubes, the same sample tube items were added. To the solution blank, 50 μL of sodium phosphate buffer 0.1 $mol.L^{-1}$ pH 7.8 was added in place of the sample. The sample and solution blank tubes had the aluminium foil removed and were placed in a wooden box containing fluorescent lights at room temperature for 10 minutes. The sample blank tubes remained with

aluminium foil, so there was no light action on them. The absorbance readings were carried out in a spectrophotometer at 560 nm using quartz cuvettes with three replicates. The results were expressed in Unit of enzyme, where 1 unit corresponds to the amount of enzyme required to inhibit 50% of the NBT photo reduction.

The activity of the catalase enzyme (CAT) was determined according to the method proposed by KRAUS et al. (1995) and modifications by AZEVEDO et al. (1998), using the soluble protein extract. The reaction was performed with three replicates composed by the addition of 25 µL of endosperm and embryo protein extract in 1 mL of potassium phosphate buffer 0.1 M pH 7.5 containing 2.5 $mL.L^{-1}$ of hydrogen peroxide 30% prepared immediately before use and protected from light. The reaction was prepared directly in quartz cuvettes and read in a spectrophotometer at 240 nm for 60 seconds at room temperature. The absorbance after 60 seconds was divided by the extinction coefficient of hydrogen peroxide (39.5 $mM.cm^{-1}$) and the results were expressed in $\mu mol.min^{-1}$.mg $protein^{-1}$.

The hydrogen peroxide quantification analysis was performed according to the methodology proposed by ALEXIEVA et al. (2001). For extraction, three replicates of 200 mg of ground embryo and ground endosperm were macerated with 3 mL of 0.1% trichloroacetic acid solution (TCA) and centrifuged at 3000 rpm for 10 minutes. The quantification reaction was composed by 200 µL of extract, 800 µL of potassium iodide 1M and 200 µL of potassium phosphate buffer solution 0.1 M pH 7.5. The reactions occurred in centrifuge microtubes (eppendorfs) and were incubated on ice for 1 hour in the dark. After this time, the samples were placed at room temperature and protected from light. Absorbance readings were performed in a spectrophotometer at 390 nm using quartz cuvettes. The results were expressed as $\mu mol.g^{-1}$.

Lipids peroxidation was determined indirectly through the quantification of malondialdehyde (MDA), according to the method proposed by CAKMAK AND HORST (1991). For extraction, three replicates of 200 mg of ground embryo and ground endosperm for each stress time were macerated with 2 mL of 0.1% trichloroacetic acid solution (TCA) and centrifuged at 3.000 rpm for 10 minutes. The quantification reaction was composed by 250 µL of extract and 1 mL of 20% trichloroacetic acid solution (TCA) containing 0.5% of thiobarbituric acid (TBA). The tubes were closed and remained in a water bath at 95 °C for 30 minutes. The reaction was then interrupted on ice for 10 minutes protected from light. The absorbance readings of the samples were performed in a spectrophotometer at 600 and 535 nm using quartz cuvettes. The difference between the two readings was divided by the molar extinction coefficient of the reaction (155 $mM.cm^{-1}$). The results were expressed as $\mu mol.g^{-1}$ of fresh weight.

The statistical analyses of the data followed the completely randomized design in factorial arrangement. The comparison of means was by Tukey test at 5% probability ($p<0.05$). Pearson correlations, Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and Partial Least Square Regression (PLS-R) were performed using software R (R CORE TEAM, 2019), through scripts developed by the research group.

4.4 RESULTS AND DISCUSSION

Analyses of variances (ANOVA) of the normal seedlings, abnormal seedlings and unviable seeds data during periods of accelerated ageing stress are shown in Table 5. For normal seedlings and unviable seeds, there was a significant interaction between the two factors (Hybrids vs. Stress time) ($p <0.05$). For the percentage of abnormal seedlings, there was only a significant effect of the stress time.

Table 5 - Summary of the analysis of variances (ANOVA) of the physiological analyses of hybrid maize seeds under periods of accelerated ageing stress

Sources of Variation	**D.F.**	**NORMAL**	**ABNORMAL**	**UNVIABLE**
			***p*-values**	
Factor 1 (Hybrids)	1	$< 0.001^{*}$	0.3009^{ns}	$< 0.001^{*}$
Factor 2 (Stress time)	4	$< 0.001^{*}$	$< 0.001^{*}$	$< 0.001^{*}$
Factor 1 vs. Factor 2	4	$< 0.001^{*}$	0.0980^{ns}	$< 0.001^{*}$
Residuals	30	-	-	-

D.F.: Degrees of Freedom.
*Significant at 5% probability ($p<0.05$) by F test. ^{ns} Not significant at 5% probability ($p>0.05$) by F test.
Source: Elaborated by the author, 2019.

From the results of Table 6, it can be observed that up to 24 hours, stress at 45 °C and saturated relative humidity did not reduce the percentage of normal seedlings for both hybrids. However, from 48 hours, there was a significant reduction in the percentage of normal seedlings for H2. The percentages were 74 % and 24 % of the normal seedlings for 48 and 72 hours, respectively for this hybrid. Hybrid 1 did not change the percentage of normal seedlings during all the stress periods, maintaining the value above 90%.

During deterioration, the seeds undergo first a biochemical deterioration followed by physiological deterioration until a rapid decline in normal and abnormal seedling percentages and an increase in the number of dead seeds (DELOUCHE; BASKIN, 1973; BEWLEY et al., 2013). Reducing the percentage of normal seedlings is one of the specific consequences of the deterioration process. This is because in conditions of high temperature and relative humidity around 100%, there is an increase in the incidence of seedling abnormalities caused by deterioration, which can progress to the point where the viability of the seeds is totally lost (DELOUCHE; BASKIN, 1973; BEWLEY et al., 2013). In relation to the percentage of

abnormal seedlings, an increase of the value with the increase of the stress period was observed, regardless of the hybrid (Table 6).

Table 6 - Percentages of normal seedlings, abnormal seedlings and unviable seeds of maize hybrids during the stress by accelerated ageing.

NORMAL SEEDLINGS (%)						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	97 aA[1]	97 aA	96 aA	95 aA	93 aA	96
H2 – 30F53VYH	100 aA	99 aA	98 aA	74 bB	24 bC	79
Mean	99	98	97	85	59	88
ABNORMAL SEEDLINGS (%)						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	1	0	2	2	4	2
H2 – 30F53VYH	0	1	2	7	4	3
Mean	1 C	1 C	2 B	5 A	4 A	3
UNVIABLE SEEDS (%)						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	2 aA	3 aA	2 aA	3 bA	3 bA	3
H2 – 30F53VYH	0 aC	0 aC	0 aC	19 aB	72 aA	18
Mean	1	2	1	11	38	11

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

The main effect of the deterioration caused by stress was observed through the increase of unviable seeds in the low-vigour hybrid starting from 48 hours after the beginning of the stress. The results of Table 6 show that there was no significant statistical difference ($p<0.05$) between the hybrids up to the 24 hour. From that moment, the hybrid of low vigour (H2) presents a significant increase in the percentage of unviable seeds of 19 % and 72 % for the periods of 48 and 72 hours, respectively. These results demonstrate that there are differences in the tolerance to ageing between the two maize cultivars. The highest tolerance was observed for the high-vigour hybrid (H1) starting from 48 hours, and in the short period of stress (from 0 to 24 hours), there was no difference between hybrids. The low-vigour hybrid seeds rapidly lost their ability to germinate after 48 hours of stress.

When the external exposure conditions to the genotypes were the same, the percentages of normal seedlings, abnormal seedlings and unviable seeds were determined by changes in the intrinsic characteristics of the seed. From the results, it was observed that the ageing in the short period (from 0 to 24 hours) did not cause increase effect in the production of abnormal seedlings and unviable seeds for both hybrids. However, as the ageing period increased, the deterioration process was accelerated in the low-vigour hybrid, increasing the percentage of abnormal seedlings and loss of seed viability.

Table 7 shows the analyses of variances (ANOVA) for the electrical conductivity (EC) test and for the moisture degree (MD) of the embryo and endosperm. There were a significant interaction between the two factors (Hybrids vs. Stress time) ($p<0.05$) for the EC test and the MD of the endosperm. For MD of the embryo, there were significant effect for hybrid and for stress time, separately.

Table 7- Summary of the analyses of variances (ANOVA) for the electrical conductivity (EC) and moisture degree (MD) of the embryo and endosperm of hybrid maize seeds under accelerated ageing stress.

Sources of Variation	**D.F.**	**EC**	**D.F**	**MD Embryo**	**MD Endosperm**
		***p*-values**		***p*-values**	
Factor 1 (Hybrids)	1	$< 0.001^{*}$	1	0.0055^{*}	$< 0.001^{*}$
Factor 2 (Times stress)	4	$< 0.001^{*}$	4	$<0.001^{*}$	$< 0.001^{*}$
Factor 1 x Factor 2	4	$< 0.001^{*}$	4	0.537^{ns}	$< 0.001^{*}$
Residuals	20	-	10	-	-

D.F.: Degrees of Freedom.
*Significant at 5% probability ($p<0.05$) by F test. ^{ns} Not significant at 5% probability ($p>0.05$) by F test.
Source: Elaborated by the author, 2019.

The results show that in the short period of stress (from 0 to 12 hours) there was no difference between the hybrids, being both tolerant to the accelerated ageing condition (Table 8). However, after 24 hours, H1 demonstrated a higher tolerance while, from this moment on, the H2 demonstrated a gradual increase in the electrical conductivity value of the soaking solution. The increased exposure to ageing caused the increase of solute leakage and, consequently, increased the loss of viability of low-vigour seeds (H2).

At 72 hours of stress, this value was even higher. On the other hand, the electrical conductivity value of H1 remains unchanged during the whole period of stress. The electrical conductivity of the seed imbibition solution increased with the stress period, indicating that accelerated ageing leads to degradation of the low vigour seed membrane system and increased electrolyte leaching (YUNFANG et al., 2006). Thus, the electrical conductivity test is an indirect measure of the integrity of the membranes and is considered one of the first symptoms of deterioration and, therefore, can be used as a method to evaluate the seeds vigour (FESSEL et al., 2006; MARCOS-FILHO, 2015).

Table 8 - Results of the electrical conductivity (μS.cm^{-1}.g seed^{-1}) of hybrid maize seeds during 0, 12, 24, 48 and 72 hours of stress by accelerated ageing.

Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	12.0 aA[1]	12.4 aA	10.8 bA	11.2 bA	11.0 bA	11.5
H2 – 30F53VYH	12.6 aC	12.1 aC	14.4 aB	14.2 aB	21.1 aA	14.9
Mean	12.3	12.2	12.6	12.7	16.0	13.2

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

Based on the results of the physiological analyses, the vigour of hybrid maize seeds was explained by the test of normal seedlings, unviable seeds and electrical conductivity. Low-vigour seeds demonstrate reduced vigour during the process due to loss of cell membrane integrity and increased conductivity. The external manifestation of these changes in the internal level was observed increasing the number of unviable seeds. On the other hand, high-vigour seeds showed greater stability of the membranes and the value of electrical conductivity during the ageing period remained unchanged. This condition was reflected externally in the percentage of normal seedlings and unviable seeds, which also remained unchanged throughout the stress.

The determination of the moisture degree of the seed structures (embryo and endosperm, separately) was performed as a control in order to verify the uniformity of condition between the hybrids and to increase the reliability of the results of the subsequent biochemical analyses, since the moisture degree may interfere in the results (MARCOS-FILHO, 2015). The ageing rate of the seeds usually increases with the moisture degree of seed, relative humidity and temperature of the exposure environment (HARMAN; MATTICK, 1976).

It was observed that, for the embryo, although there was a statistical difference ($p<0.05$) between hybrids (Table 7), this difference was only 1%, with an average of 28% humidity for H1 and 27% for H2 (Table 9). This value was sensitive to detect statistical difference, but was insignificant at the biological level. Although seeds do not come into direct contact with water during accelerated ageing, they absorb moisture gradually during the exposure time to saturated relative humidity and high temperatures due to the hygroscopic characteristic of the seeds.

For the endosperm, there was a significant interaction between the two factors (Hybrids vs. Stress time) ($p<0.05$) (Table 7). The largest difference (2%) between the hybrids was detected in the period of 24 hours of stress, being 18 % humidity for H1 and 16 % for H2 (Table 9). In the same way of the embryo, this difference is merely mathematical and insignificant at the biological level. Therefore, subsequent analyses were not compromised by the difference in moisture between the hybrids, for both the embryo and the endosperm, since

it is recommended that the difference in the moisture percentage of the samples does not exceed 2 % (MARCOS-FILHO, 1999; COIMBRA et al., 2009; SENA et al., 2017; SANTOS et al., 2017).

Table 9 - Results of the moisture degree (MD) of embryo and endosperm of hybrid maize seeds during 0, 12, 24, 48 and 72 hours of stress by accelerated ageing.

MOISTURE DEGREE - MD (%) - EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	11	22	27	37	41	28 a
H2 – 30F53VYH	11	20	26	35	41	27 b
Mean	11 E[1]	21 D	27 C	36 B	41 A	27
MOISTURE DEGREE - MD (%) - ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	12 bD	16 aC	18 aB	20 aA	20 aA	17
H2 – 30F53VYH	13 aD	16 aC	16 bB	19 bA	19 bA	17
Mean	13	16	17	20	20	17

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

Although the seeds do not have direct contact with the water during the accelerated ageing period, the intrinsic hygroscopic characteristic of the seeds promotes the absorption of sufficient moisture to initiate the activation of some enzymatic metabolism and hydrolytic reactions (BEWLEY et al., 2013). Table 10 shows the analyses of variances (ANOVA) of the biochemical data performed on the embryo and on the endosperm of hybrid maize seeds during 0, 12, 24, 48 and 72 hours of stress by accelerated ageing. For the starch variable, there was no significant interaction between the two factors for both embryo and endosperm. In the endosperm, there was only significant effect of the stress time and in the embryo, there were an effect of the stress time and hybrid, separately ($p<0.05$).

Table 10- Summary of the analyses of variances (ANOVA) for the biochemical data of the embryo and endosperm of hybrid maize seeds under accelerated ageing stress. Starch (SCH); Total Soluble Sugar (TSS); α-amylase (AMY); Total Soluble Protein (TSP), Superoxide Dismutase (SOD); Catalase (CAT); Hydrogen peroxide (H2O2); Malondialdehyde (MDA).

ANOVA - ENDOSPERM									
Sources of Variation	**D.F.**	**SCH**	**TSS**	**AMY**	**TSP**	**SOD**	**CAT**	**H_2O_2**	**MDA**
		***p*-values**							
Factor 1 (Hybrids)	1	0.8689^{ns}	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	0.2601^{ns}	$<0.001^{*}$	$<0.001^{*}$
Factor 2 (Stress time)	4	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	0.002^{*}	0.0022^{*}	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$
Factor 1 x Factor 2	4	0.081^{ns}	0.0012^{*}	$<0.001^{*}$	0.002^{*}	0.2049^{ns}	0.0169^{*}	$<0.001^{*}$	0.4988^{ns}
Residuals	20	-	-	-	-	-	-	-	-
ANOVA - EMBRYO									
Sources of Variation	**D.F.**	**SCH**	**TSS**	**AMY**	**TSP**	**SOD**	**CAT**	**H_2O_2**	**MDA**
		***p*-values**							
Factor 1 (Hybrids)	1	$<0.001^{*}$	$<0.001^{*}$	0.0019^{*}	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	0.696^{ns}
Factor 2 (Stress time)	4	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$	$<0.001^{*}$
Factor 1 x Factor 2	4	0.3735^{ns}	$<0.001^{*}$	0.1304^{ns}	$<0.001^{*}$	0.0038^{*}	0.0061^{*}	$<0.001^{*}$	$<0.001^{*}$
Residuals	20	-	-	-	-	-	-	-	-

D.F.: Degrees of Freedom.

* Significant at 5% probability ($p<0.05$) by F test. ^{ns} Not significant at 5% probability ($p>0.05$) by F test. Source: Elaborated by the author, 2019.

The energy required to maintain the physiological and biochemical processes comes from the hydrolysis and mobilisation of carbohydrates, lipids and protein present on reserves tissues, since at that moment seeds do not have structures to absorb nutrients and to make photosynthesis (COELHO; BENEDITO, 2008; BEWLEY et al., 2013; YU et al., 2014). From the results, it was observed that there was no statistical difference between the starch content of the endosperm in high and low vigour hybrids (Table 11).

There was hydrolysis of the starch, verified by the decrease in the starch content starting from 24 hours of stress, regardless of the hybrid. On the other hand, in relation to the embryo starch content, there was a significant difference between the hybrids ($p<0.05$), being 32.6 % and 22.9 % of starch for the high vigour (H1) and low vigour (H2), respectively (Table 11). In the same way as the endosperm starch, the embryo starch underwent a gradual decrease caused by hydrolysis from the period of 24 hour for both hybrids.

From the results, it can be verified that seeds more tolerant to stress have higher levels of starch in the embryo, while more sensitive seeds have lower contents of this component. SANTOS et al. (2013) and NERLING et al. (2018) found no significant positive correlation between seed vigour and starch content.

Table 11 - Percentages of starch in endosperm and embryo of hybrids during stress by accelerated ageing.

STARCH (%) - ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	76.4	75.4	65.8	55.6	50.1	64.7
H2 – 30F53VYH	76.5	68.3	64.9	59.0	55.7	64.9
Mean	76.5 A[1]	71.8 A	65.4 B	57.3 C	52.9 C	64.8
STARCH (%) - EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	38.2	37.8	33.3	27.5	26.0	32.6 a
H2 – 30F53VYH	29.1	29.0	21.5	20.8	14.2	22.9 b
Mean	33.7 A	33.4 A	27.4 B	24.2 BC	20.1 C	27.8

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

For total soluble sugar (TSS), there was a significant interaction between the factors ($p<0.05$) (Table 10). Thus, it was observed that in almost all periods of stress, H1 showed superiority in the TSS of the endosperm when compared to H2, except for the period of 72 hours, where the content was similar between them (Table 12). Regarding the behaviour of this reserve component during stress, it was observed that there was a gradual decrease of the total soluble sugar content in the endosperm after 48 hours for both hybrids. This result may be associated to the carbohydrate demand when the seeds were submitted to the ageing condition.

The soluble sugar content was approximately 10 times higher in the embryo than in the endosperm. The results of Table 12 show that for H1, there was an increase in the TSS content after 24 hours from the hydrolysis of the starch, followed by reduction after 48 and 72 hours. For H2, the increase in the TSS content was after 12 hours, and only then the content was higher than H1. Based on the results of changes in the behaviour of soluble sugars, a higher TSS content was observed in the embryo for H1, except at the 12 hours stress period, suggesting a greater efficacy in carbohydrate metabolism in high vigour seeds during the accelerated ageing.

Table 12- Results of total soluble sugar in endosperm and embryo of hybrids during accelerated ageing.

TOTAL SOLUBLE SUGAR (mg.g^{-1} DW) - ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	7.0 aA[1]	7.9 aA	7.3 aA	5.4 aB	4.7 aB	6.5
H2 – 30F53VYH	5.6 bA	6.1 bA	6.5 bA	4.7 bB	4.4 aB	5.5
Mean	6.3	7.0	6.9	5.0	4.6	6.0
TOTAL SOLUBLE SUGAR (mg.g^{-1} DW) - EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	88.7 aB	80.0 bC	98.4 aA	78.9 aC	60.5 aD	81.3
H2 – 30F53VYH	73.8 bB	87.4 aA	78.6 bB	70.8 bB	39.0 bC	69.9
Mean	81.2	83.7	88.5	74.8	49.8	75.6

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

Although the hydrolysis of starch started after 24 hours, the increase in the total soluble sugar content was not observed, because at that moment the use is instantaneous to maintain the metabolism and try to overcome the stress imposed on the seeds. There was a trend of reduction in the TSS content with the increase of the stress period for the two hybrids. This reduction, as well as in the endosperm, was associated with the use of this component as a source of energy and as a substrate for respiration.

A relationship was then made between the initial content of the starch in the embryo and after 72 hours of stress. It was observed that, for the high-vigour hybrid, there was a maintenance rate of 68.1 % starch, while for the low-vigour hybrid the maintenance rate was 48.8 %. In relation to the total soluble sugars (TSS), it was observed that, despite having a higher starch maintenance rate, H1 presented higher TSS content. These sugars may have helped to overcome stress, while in H2 there was more starch hydrolysis, because the maintenance rate was lower, but the sugar content was lower than in H1. It is suggested that this genotype may have consumed more carbohydrates in the respiratory processes, trying to overcome stress or having lost them due to the lower integrity of the membranes.

The results obtained in this experiment showed that the hybrid with higher total soluble sugar content had a higher tolerance to stress. The same results were found by NERLING et al., (2018), who used inbred lines and hybrid seeds to study the association of biochemical components with seed vigour. These authors reported that the higher sensitivity in the accelerated ageing test may be associated with lower levels of soluble sugars present in the seeds. In addition, some authors have suggested that some oligosaccharides (e.g. raffinose family oligosaccharides), have essential functions in membrane stability, in combating oxidative stress caused by the presence of reactive oxygen species and providing a source of carbohydrate for respiration process (LI et al., 2011; BEWLEY et al., 2013; KEUNEN et al., 2013; SANTOS et al., 2017; NERLING et al., 2018).

Our results also corroborate with the results previously reported by HAN et al., (2017) who found further changes in carbohydrates in the embryo during the germination of wheat seeds. Thus, this structure provides a greater energetic amount for synthesis of other compounds and other metabolic demands through glycolysis, when compared to the endosperm. The availability of soluble sugars necessary to maintain the viability of the seed is dependent on the action of the amylase enzymes. The supply of substrate to the respiration process is dependent on the soluble sugar content. Reducing the content of this component may lead to a reduction in the availability of substrate for respiration (SANTOS et al., 2017), leading to an increase in the number of unviable seeds.

The ANOVA results of the α-amylase activity are shown in Table 10. It was observed that for the endosperm there was significant interaction between factors 1 and 2, different from the embryo, which had a significant effect of the factors, separately. It can be observed that, for the activity of the enzyme in the endosperm, there was no difference between the hybrids until the period of 48 hours (Table 13).

Table 13- Activity of α-amylase enzyme in the endosperm and in the embryo of hybrid maize seeds during accelerated ageing.

α-AMYLASE (Units of enzyme.kg^{-1} FW) - ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	1.0 aC[1]	1.1 aBC	1.4 aAB	1.5 aA	1.0 bC	1.2
H2 – 30F53VYH	1.0 aC	1.2 aB	1.4 aB	1.4 aB	1.8 aA	1.4
Mean	1.0	1.2	1.4	1.5	1.4	1.3
α-AMYLASE (Units of enzyme.kg^{-1} FW) - EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	6.8	9.0	10.8	7.3	8.0	8.4 b
H2 – 30F53VYH	7.9	9.8	10.6	7.8	9.4	9.1 a
Mean	7.4 C	9.4 B	10.7 A	7.6 C	8.7 B	8.8

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

After 72 hours, H2 presented higher activity when compared to H1, with 1.8 and 1.0 units of enzyme.kg^{-1} FW, respectively. For the high-vigour hybrid (H1), there was a gradual increase in the enzymatic activity in the endosperm with the increase of the stress period up to 48 hours, decreasing after 72 hours. The highest enzymatic activity was observed in the periods of 24 and 48 hours (Table 13).

For the low-vigour hybrid, the gradual increase behaviour of the enzymatic activity was also observed. However, the peak activity only occurred after 72 hours (Table 13). This decreasing behaviour of the endosperm starch can be explained by the amount of water contained in the seeds, which was sufficient to activate the hydrolysis of the starch granules stored by the action of the α-amylase enzyme to provide soluble sugars to be used by the embryo during the period of stress. Analysing the changes in α-amylase activity in the embryo, the highest enzyme activity, on average, was observed for H2 samples (Table 13). There was a peak activity after 24 hours of stress, regardless of the hybrid.

The ANOVA results of the total soluble protein data are shown in table 10. It was observed that there was a significant interaction between the factors (hybrid and the stress time) for both the endosperm and the embryo. It was observed that the highest levels of total soluble protein (TSP) were found in the embryo, when compared to the endosperm, for the two hybrids (Table 14).

Table 14- Results of total soluble protein in the endosperm and in the embryo of hybrid maize seeds during the stress periods by accelerated ageing.

TOTAL SOLUBLE PROTEIN (mg.g^{-1} FW) - ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	3.2 aA[1]	2.3 aB	2.7 aB	2.2 aB	2.4 aB	2.6
H2 – 30F53VYH	2.3 bA	2.2 aA	2.0 bA	2.4 aA	2.0 aA	2.2
Mean	2.8	2.2	2.4	2.3	2.2	2.4
TOTAL SOLUBLE PROTEIN (mg.g^{-1} FW) - EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	41.3 aA	40.2 aAB	36.4 aB	30.6 aC	28.2 aC	35.3
H2 – 30F53VYH	34.2 bA	23.1 bB	24.9 bB	22.2 bB	22.2 bB	25.3
Mean	37.8	31.6	30.6	26.4	25.2	30.3

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

The greatest changes were observed in the embryo, where H1 presented the highest total soluble protein content. The main function of the hydrolysis of seed proteins in stress situations prior to seedling formation is the increased availability of amino acids to be used in the synthesis of new enzymes (RAJJOU et al., 2012; BEWLEY et al., 2013; SANTOS et al., 2017). Thus, in

the accelerated ageing condition, hybrids of higher vigour present higher release of amino acids from the protein hydrolysis to be used in the synthesis enzymes.

Differences in initial and final TSP contents in the embryo were calculated to determine the percent reduction of this component during stress. There was a trend of decrease in the content of this component for both cultivars with 68.3 and 64.9 % of degradation after 72 hours for H1 and H2, respectively. Even with degradation by the high-vigour hybrid, the TSP content in the embryo was higher than the low-vigour, evidencing that H1 had a greater efficacy in the use of the soluble proteins during the accelerated ageing. Proteins are formed by a set of amino acids linked by peptide bonds. With the absorption of moisture by the seed, occurs the activation of hydrolytic enzymes responsible for the degradation of proteins, releasing amino acids for the synthesis of new proteins and enzymes (BEWLEY et al., 2013; SANTOS et al., 2017).

In addition, amino acids may have the amine radical removed to be used as a substrate in energy production reactions (RAJJOU et al., 2012; BEWLEY et al., 2013). Thus, the higher soluble protein content resulted in the higher seed vigour, and consequently, in the stress tolerance when compared to the vigour of seeds with lower content of this component. However, these results are in disagreement with those obtained by SANTOS et al., (2017) and NERLING et al., (2018), who found absence of correlation between protein contend and vigour of maize seeds. In a study by XIN et al. (2011) using maize seeds, enzymes involved in energy production metabolism (glycolysis, tricarboxylic acid cycle, electron transport chain and oxidative phosphorylation) were the largest group of proteins that underwent changes during accelerated ageing, suggesting importance of the roles of mobilization of stored carbohydrates and energy supply during ageing and the expression of seed vigour.

The protein profile of the embryo and the endosperm during stress can be observed in Figure 4. The largest differences in band intensity were found in the regions of 25; 50-75 kDa for the endosperm and 10; 37-50; 50-75 and 75-100 kDa for the embryo. Figure 5 shows the intensity of the protein bands in the endosperm, where H1 had greater protein expression at 25 kDa and at 50-75 kDa for all periods of stress, especially at 0 hours, when compared to H2. The figure 5 also indicates that there was lower expression of these proteins in the H2 after 24 hours of stress, verified by the reduction in the intensity of the bands in that region.

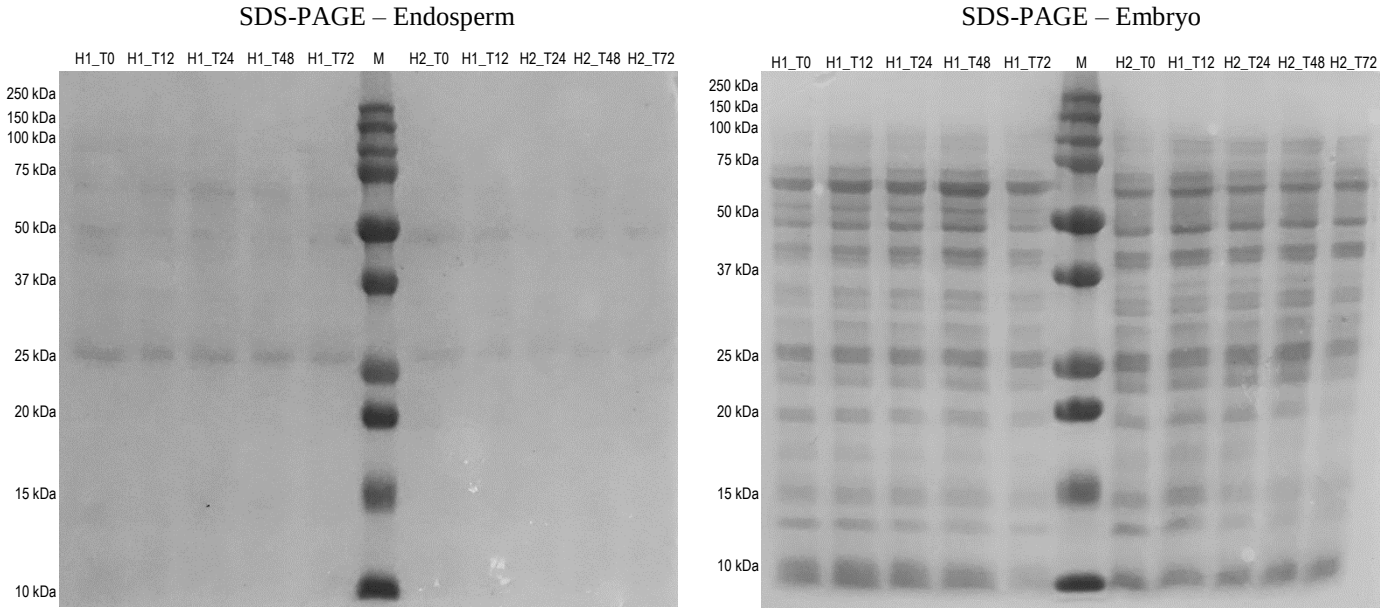
Figure 6 shows the intensity of the gel bands of the embryo. In the region of 10 kDa, there was a higher intensity for the hybrid with less vigour at time 0, 48 and 72 hours. These proteins may be associated with antioxidant enzymes, such as CAT, SOD, or other proteins. Thus, we suggest further studies of proteomics to help define which proteins are present in this

region, since it was demonstrated in this study that they undergo changes during the period of exposure to stress.

For the bands located in the region between 37 and 50 kDa, the results were very interesting, where H2 presented higher bands intensity when compared to H1, mainly in the periods of 12 and 72 hours (Figure 4 (right) and Figure 6).

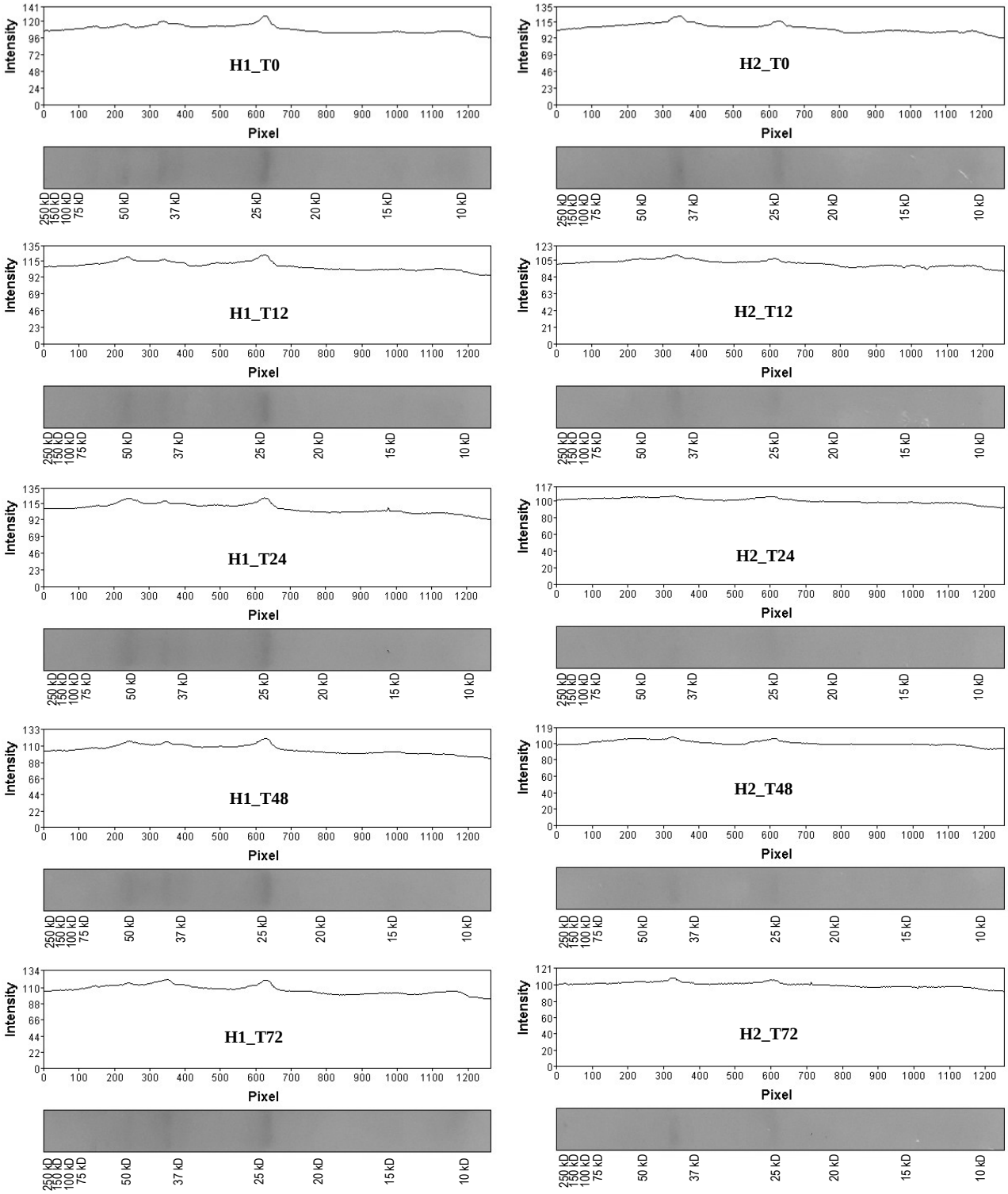
On the other hand, in the region between 50-75 kDa, the highest intensities were observed for H1, mainly in the period of 48 hours. We suggest further studies in these regions to identify the proteins associated with this molecular weight. In the region between 75-100 kDa, there was higher protein expression for H2, especially in the periods of 12, 24 and 48 hours.

Figure 4 - Electrophoretic protein profile of the endosperm and embryo of hybrid maize seeds during stress periods by accelerated ageing.



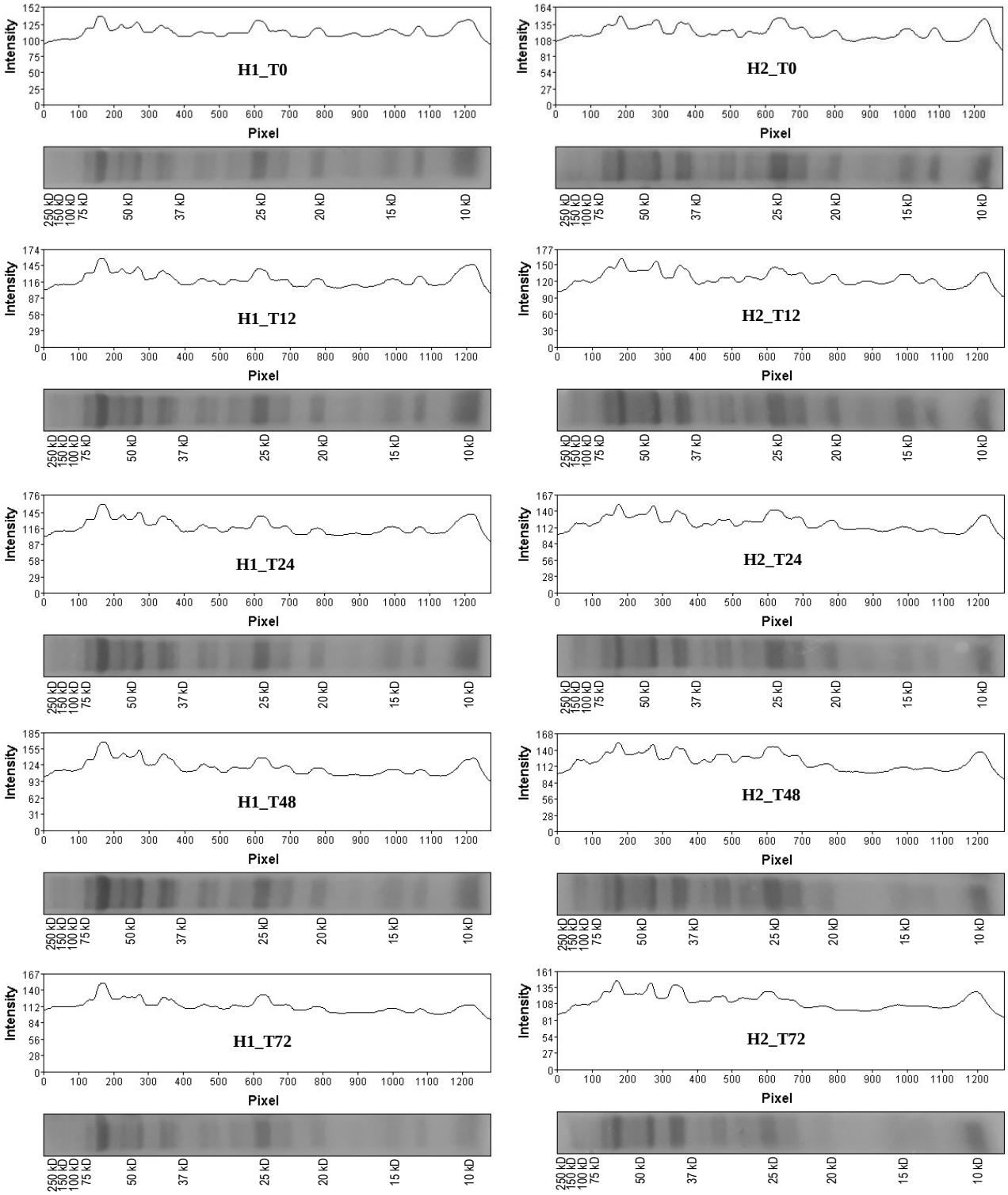
kDa - kilodaltons
Source: Elaborated by the author, 2019.

Figure 5- Intensity of the electrophoretic bands of the endosperm of the high and low vigour hybrids identified by the software Gel Analyzer.



Source: Elaborated by the author, 2019.

Figure 6 - Intensity of the electrophoretic bands of the endosperm of the high and low vigour hybrids identified by the software Gel Analyzer.



Source: Elaborated by the author, 2019.

The ANOVA results of the enzymatic activity of superoxide dismutase (SOD) are shown in Table 10. There was no significant interaction between the factors for the endosperm, which did not happen for the embryo. The results of table 15 show that the activity of the enzyme was much higher than in the endosperm than in the embryo. In addition, there was a trend of increased activity with increased stress, regardless of the hybrid. By the analysis, it was observed that the stress caused greater activity in the hybrid of low vigour, associated to the greater sensitivity of this hybrid that used the antioxidant mechanism as an attempt to overcome the stress.

Table 15- Superoxide dismutase (SOD) activity in the endosperm and in the embryo of hybrid maize seeds during stress by accelerated ageing.

SOD ACTIVITY (Units of enzyme.mg prot^{-1}) – ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	775.8	944.3	1089.9	1123.6	1063.3	999.4 b
H2 – 30F53VYH	1056.7	1111.5	1218.9	1112.8	1254.6	1150.9 a
Mean	916.2 B[1]	1027.9 AB	1154.4 A	1118.2 A	1159.0 A	1075.1
SOD ACTIVITY (Units of enzyme.mg prot^{-1}) – EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	60.6 bC	62.4 bC	68.9 bBC	82.1 bAB	88.9 bA	72.6
H2 – 30F53VYH	73.4 aB	101.0 aA	108.4 aA	113.2 aA	112.6 aA	101.7
Mean	67.0	81.7	88.6	97.6	100.8	87.1

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

The activity of the enzymes superoxide dismutase and catalase are widely studied under different stress conditions because they are known antioxidant agents, performs the function of removing reactive species of oxygen that are toxic to the cells, transforming the H_2O_2, for example, into water and oxygen (SANTOS et al., 2015). As in the endosperm, the activity of the SOD enzyme in the embryo was higher in the low-vigour hybrid. There was a trend of increased activity for both hybrids, but for H1, this increase was gradual during stress, while for H2, after 12 hours there was an increase in activity, which remained high up to 72 hours.

In the H1 embryo, the enzyme activity ranged from 60.6 to 88.9 units of enzyme.mg prot^{-1}, that is, increased 28.3 units of enzyme.mg prot^{-1} after 72 hours of stress (Table 15). However, for the H2 embryo, the SOD activity ranged from 73.4 to 112.6 units of enzyme.mg prot^{-1}. Thus, an increase of 39.2 units of enzyme.mg prot^{-1} occurred after 72 hours of ageing. Thus, the results indicate that the antioxidant metabolism of the low-vigour hybrid was activated in the attempt to overcome stress, when compared to H1 due to higher sensitivity. For the high-vigour hybrid, the stress was not severe enough to require more activity of this system.

Another important enzyme to combat oxidative stress in seeds is catalase (CAT). The results of Table 10 show that there was significant interaction for both the endosperm and the embryo. Greater enzymatic activity was observed in the endosperm than in the embryo, as well as the behaviour of SOD. There was a gradual increase in CAT activity in the high vigour seeds endosperm (Table 16).

Table 16- Catalase (CAT) activity in the endosperm and in the embryo of hybrid maize seeds during stress by accelerated ageing.

CAT ACTIVITY (μmol.min^{-1}.mg prot^{-1}) – ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	276.3 aC[1]	294.4 aC	319.6 aBC	394.5 aB	475.6 aA	352.1
H2 – 30F53VYH	283.4 aB	332.3 aB	340.2 aB	317.7 bB	421.5 bA	339.0
Mean	279.8	313.4	329.9	356.1	448.6	345.6
CAT ACTIVITY (μmol.min^{-1}.mg prot^{-1}) – EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	81.7 bA	94.9 bA	104.3 bA	110.4 bA	95.3 bA	97.3
H2 – 30F53VYH	119.8 aC	177.0 aB	167.9 aB	214.1 aA	192.7 aAB	174.3
Mean	100.8	136.0	136.1	162.2	144.0	135.8

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

After 48 hours of stress, the activity of the enzyme in the endosperm of H1 was significantly higher when compared to H2. The enzymatic activity ranged from 276.3 to 475.6 μmol.min^{-1}.mg prot^{-1} for H1 and 283.4 to 421.5 μmol.min^{-1}.mg prot^{-1} for H2. There was an increase of 199.3 μmol.min^{-1}.mg prot^{-1} for H1 versus 138.1 μmol.min^{-1}.mg prot^{-1} for H2. Thus, higher efficiency of CAT was observed in the high vigour seed endosperm.

On the other hand, in the embryo the activity of the enzyme in the hybrid of low vigour was significantly higher in all periods of stress, as was observed for the enzyme SOD (Table 16). These same results were found by SANTOS et al. (2015) evaluating the catalase activity in lines maize seeds during temperature stress, were seeds with higher vigour showed lower activity. This can be explained by the fact that stress was not as drastic for more vigorous genotypes, so there was no need to activate stress-fighting mechanisms in the same way as the more sensitive hybrids that used their mechanisms to try to overcome the adverse condition.

There was a large increase in CAT activity in the H2 embryo, from 119.8 to 192.7 μmol.min^{-1}.mg prot^{-1}, which means that there was an increase of 72.9 μmol.min^{-1}.mg prot^{-1}. For H1, the enzymatic activity in the embryo started from 81.7 to 95.3 μmol.min^{-1}.mg prot^{-1}, that is, an increase of 13.6 μmol.min^{-1}.mg prot^{-1}. As observed by previous analyses discussed in this research, H1 presents greater tolerance to stress because of its high vigour.

The ANOVA results of hydrogen peroxide content in the embryo and endosperm of maize seeds are shown in Table 10. There was significant interaction ($p<0.05$) between the factors (Hybrids vs. Stress time) for the two seed structures. The results of hydrogen peroxide content for the endosperm show that, up to 24 hours the H_2O_2 content was significantly higher in H2 ($p<0.05$) (Table 17). The higher concentration of H_2O_2 in the cells is directly associated with the deterioration process, due to the oxidation of the cells caused by the free radicals (SANTOS et al., 2015).

It was also observed that the activity of the enzymes responsible for combating this component in the endosperm (SOD and CAT) increased during the stress period for both hybrids. Thus, hydrogen peroxide content tended to decrease as the stress period increased due to enzymatic activity. In the embryo the H_2O_2 content was significantly higher for the low-vigour hybrid, except in the 48-hour period, where the content was similar between H1 and H2.

Free radicals released during stress conditions culminate in destructive reactions, damaging mainly cell membranes. Thus, the functioning of mitochondria, where the chemical reactions of respiration occur, is compromised, along with the supply of energy and secondary compounds for the synthesis of proteins (DELOUCHE; BASKIN, 1973; McDONALD, 1999; BEWLEY et al., 2013; MARCOS-FILHO, 2015). In addition, there is an increase in leaching of electrolytes due to loss of membrane integrity (DELOUCHE; BASKIN, 1973; McDONALD, 1999; BEWLEY et al., 2013; MARCOS-FILHO, 2015).

Table 17- Hydrogen peroxide content (H_2O_2) in the endosperm and in the embryo of hybrid maize seeds during stress by accelerated ageing.

HYDROGEN PEROXIDE CONTENT (µmol.g^{-1}) – ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	0.67 bA[1]	0.54 bB	0.57 bB	0.56 aB	0.54 aB	0.63
H2 – 30F53VYH	0.72 aA	0.71 aA	0.63 aB	0.54 aC	0.54 aC	0.58
Mean	0.69	0.62	0.60	0.55	0.54	0.60
HYDROGEN PEROXIDE CONTENT (µmol.g^{-1}) – EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	4.51 bAB	5.21 bA	3.90 bBC	3.18 aC	2.27 bD	3.81
H2 – 30F53VYH	7.05 aA	6.21 aA	4.54 aB	3.21 aC	2.99 aC	4.80
Mean	5.78	5.71	4.22	3.20	2.63	4.30

[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

Lastly, the lipid peroxidation was verified indirectly through the quantification of malondialdehyde content (MDA), one of the products of this reaction. The ANOVA results of Table 10 show that there was no significant interaction between the factors in the endosperm, only hybrid effect and stress time, separately. Table 18 shows that in the endosperm, lipid

peroxidation was higher in H2 when compared to H1. In addition, there was a gradual decrease in MDA content, regardless of the genotype. This decrease is associated with increased activity of antioxidant enzymes SOD and CAT.

The higher lipid peroxidation in the low-vigour hybrid was also observed by the electrical conductivity test after 24 hours as was demonstrated and discussed previously. Thus, it is suggested that the higher sensitivity of H2 can be explained by the greater deterioration of membranes and lipid peroxidation caused by the temperature of 45 °C and high relative humidity in ageing. On the other hand, H1 demonstrated higher membrane stability, as it did not alter the electrical conductivity during stress and had lower H_2O_2 and MDA content in the endosperm.

On the other hand, in the embryo the highest MDA content was observed in H1 in the 72 hour period (Table 18). For seeds non-stressed (T0), the highest content was observed in H2. There was a trend of decrease of the content of this component for the two hybrids due to the increase in the antioxidant enzymatic activity. Although H1 presented a higher MDA content within 72 hours, the increase in electrical conductivity was not visualized, which may indicate the absence of membrane deterioration (Table 8).

The H1 embryo may have compensated for the higher MDA content due to the higher content of total soluble sugars, which aid in increasing the stability of membranes in the stress condition. Thus, H1 was able to excel at stress, while H2 had the low vigour explained by the physiological and biochemical components, since it increased drastically the number of unviable seeds because it did not withstand the adverse condition imposed on them.

Table 18- Lipids peroxidation through the malondialdehyde (MDA) content in the endosperm and embryo of hybrid maize seeds during accelerated ageing stress.

LIPIDS PEROXIDATION - MDA (µmol.g^{-1}) - ENDOSPERM						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	0.38	0.25	0.29	0.20	0.22	0.27 b
H2 – 30F53VYH	0.41	0.34	0.38	0.31	0.29	0.35 a
Mean	0.40 A[1]	0.30 BC	0.34 AB	0.26 C	0.26 C	0.31
LIPIDS PEROXIDATION - MDA (µmol.g^{-1}) - EMBRYO						
Hybrid	T0	T12	T24	T48	T72	Mean
H1 – DKB230PRO3	5.54 bA	5.36 aA	3.28 aB	2.71 aBC	2.29 aC	3.83
H2 – 30F53VYH	6.46 aA	5.93 aA	3.33 aB	2.11 aC	1.09 bD	3.78
Mean	6.00	5.65	3.31	2.41	1.69	3.81

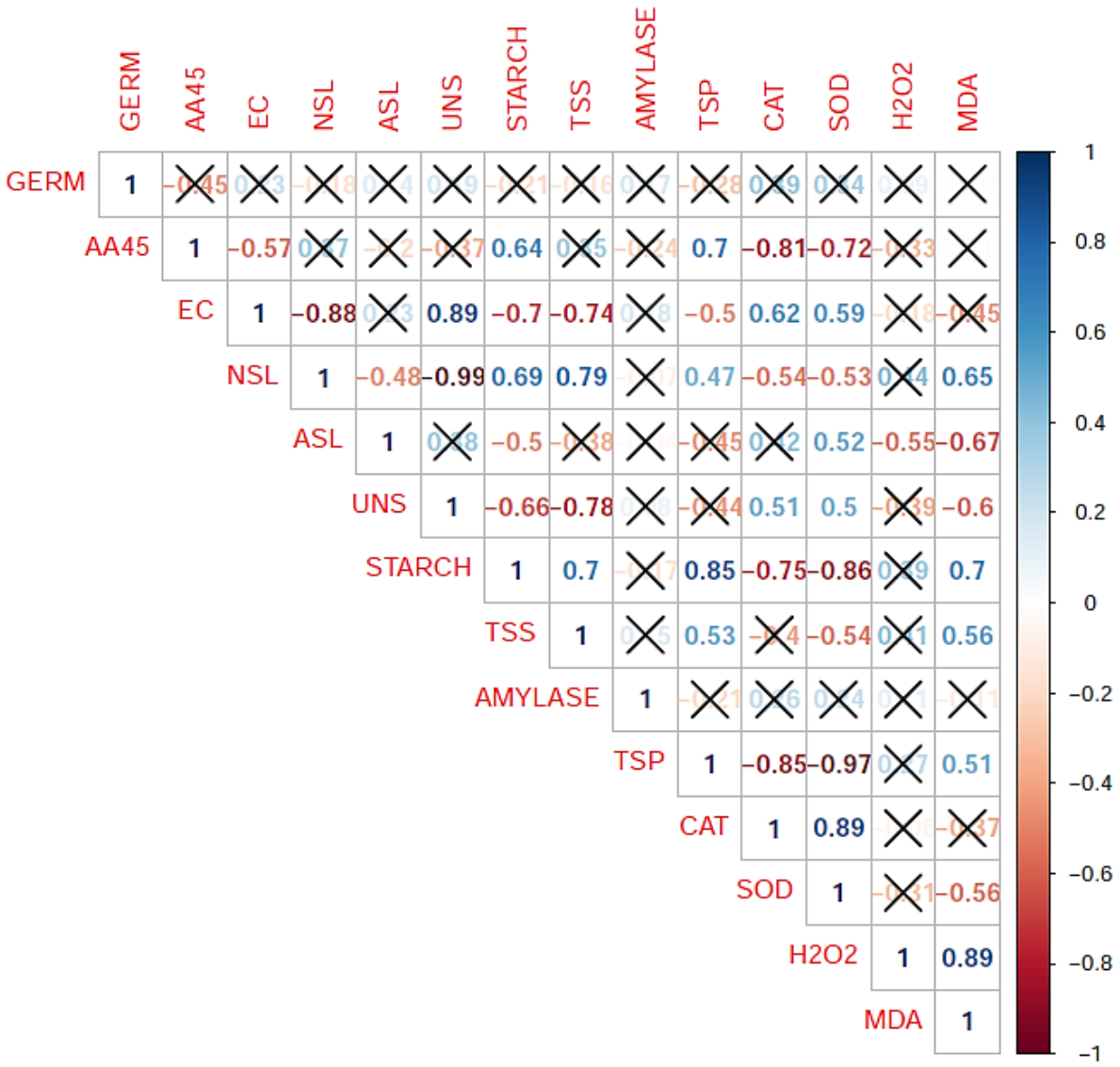
[1]Means followed by the same letter do not differ statistically from each other by the Tukey test at 5% probability ($p<0.05$), being uppercase in the row and lowercase in the column. Source: Elaborated by the author, 2019.

To investigate the relationships between metabolites and seed vigour, we performed Pearson correlations, PCA, HCA and PLS-R techniques. Through the Pearson correlation

coefficients of the embryo it was possible to observe that the vigour of the maize seeds was dependent on the starch content (r= +0.64) and total soluble protein (r= +0.70) (Figure 7). In addition, vigorous seeds showed lower electrical conductivity (r= -0.57), indicating that the higher the stress sensitivity, the greater the leakage of solutes due to the loss of membrane integrity. This result was also observed in a study developed by ZHANG et al. (2007), where the authors found a negative correlation between the evaluated vigour indexes and the electrical conductivity of the seed imbibition solution. The greater vigour by accelerated ageing was negatively correlated with the activity of CAT (r= -0.81) and SOD (r= -0.72). It is suggested that this behaviour occurred because the seeds more sensitive to stress had their metabolism accelerated in the attempt to overcome the adverse condition (Figure 7).

Other important relationships were observed for the variable of electrical conductivity, which had negative correlation with the percentage of normal seedlings (r= -0.88), starch content (r= -0.70), total soluble sugar (r= -0.74) and total soluble protein (r= -0.50). It can be stated from these results that seeds with higher electrical conductivity have lower contents of these compounds. On the other hand, the increase in electrical conductivity causes an increase in the percentage of unviable seeds (r= +0.89), SOD activity (r= +0.59) and CAT activity (r= +0.62). According to BAILLY et al. (1996), there is a relation between loss of seed viability and increase in permeability and loss of membranes integrity. The percentage of normal seedlings during stress was dependent on the starch content (r = +0.69), total soluble sugar (r = +0.79) and total soluble protein (r = +0.47) (Figure 7).

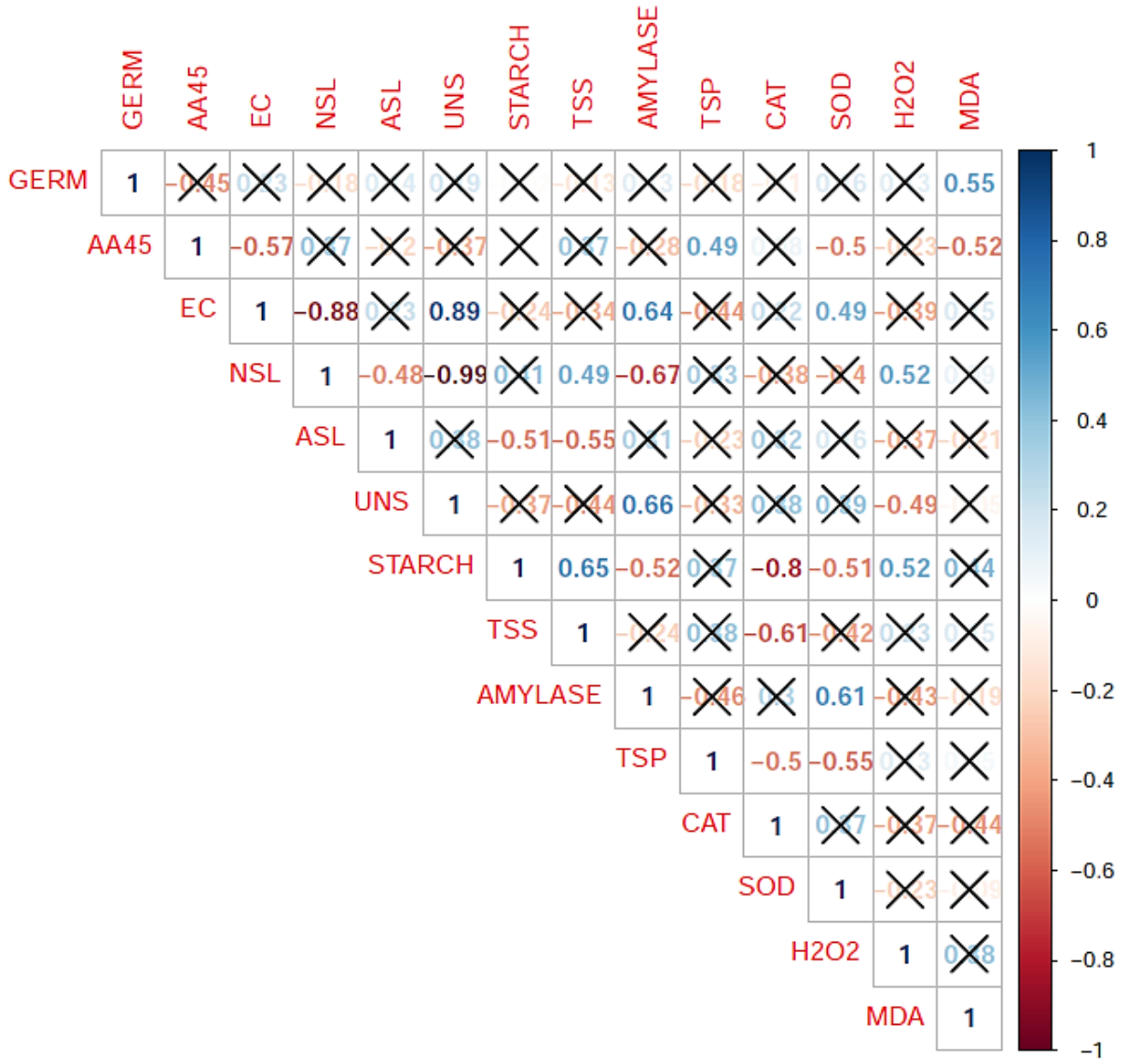
Figure 7 - Pearson correlation between physiological and biochemical analyses of embryo. The data covered by X were not significant at 1% probability (p <0.01) by the t test.



GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019.

For the endosperm, there was a negative correlation between vigour by accelerated ageing and electrical conductivity (r= -0.57), in addition to the positive correlation with total soluble protein content (r= +0.49) (Figure 8). The percentage of normal seedlings of the samples was dependent on the total soluble sugar content (r= +0.49) and the lower electrical conductivity of the soaking solution (r= -0.88) (Figure 8).

Figure 8 - Pearson correlation between physiological and biochemical analyses of endosperm. The data covered by X were not significant at 1% probability ($p < 0.01$) by the t test.

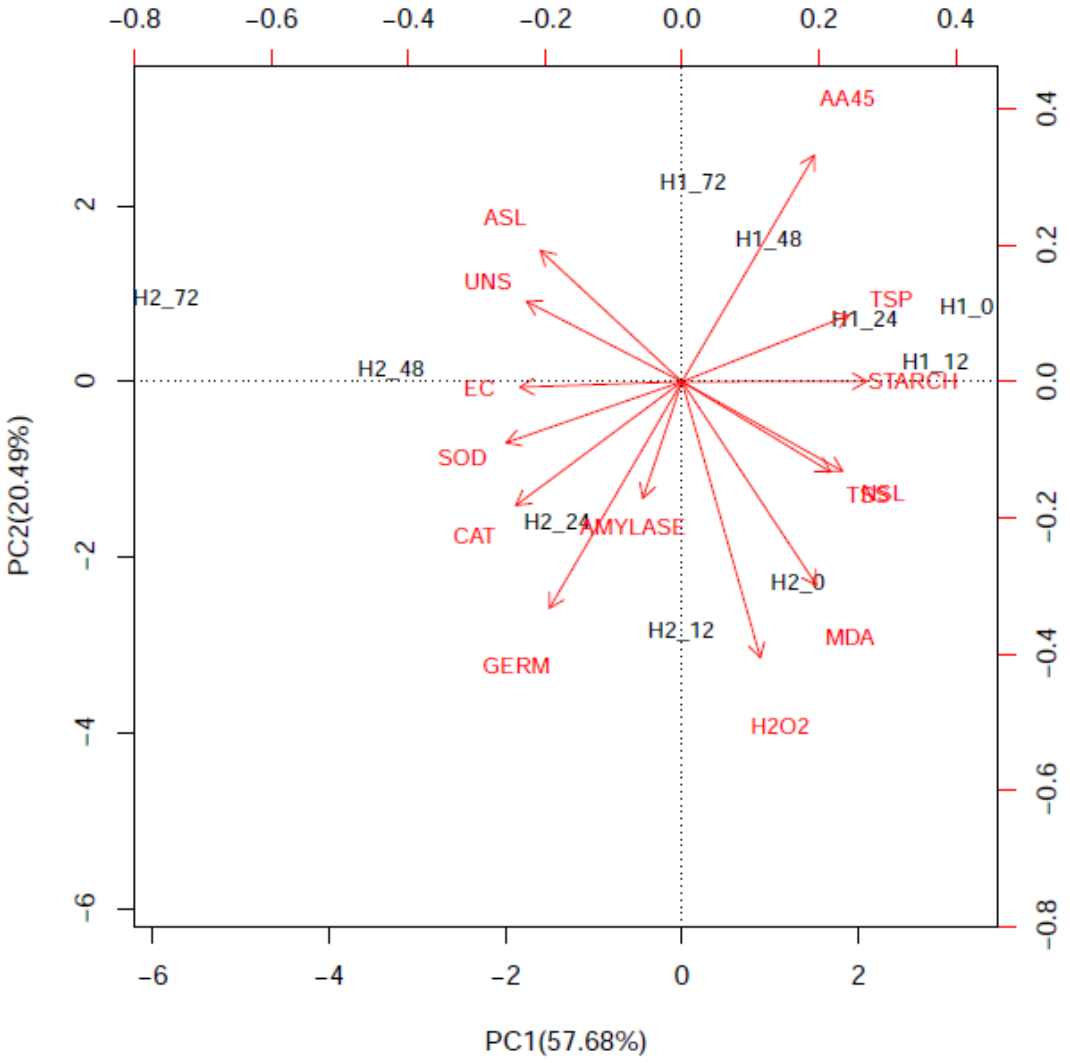


GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019.

With the purpose of detailing the results of the analyses and indicating the variables most correlated with maize seed vigour during accelerated ageing, Principal Component Analysis (PCA) of the embryo and endosperm were performed. The results obtained in the analysis of embryo show that the principal components PC1 and PC2 were responsible for capturing 57.68% and 20.49% of the data variation, respectively, totalling 78.17% (Figure 9). It can be seen in the diagram that there was a clear separation between H1 and H2 samples during stress times. All samples of H1 (0, 12, 24, 48 and 72 hours) were clustered in PC1+/PC2+

as a function of total soluble protein, starch content and higher vigour by accelerated ageing. The samples of the H2 at 0 and 12 hours were grouped in PC1+/PC2- as a function of the H_2O_2 and MDA contents. The samples of the H2 at the 24 hour time were grouped in PC1-/PC2- by the CAT and SOD variables. Finally, samples of H2 at 48 and 72 hours of stress were grouped in PC1-/PC2+ by the variables of electrical conductivity, abnormal seedlings and unviable seeds.

Figure 9 - Principal Component Analysis (PCA) of maize hybrids embryo subjected to accelerated ageing for 0, 12, 24, 48 and 72 hours.

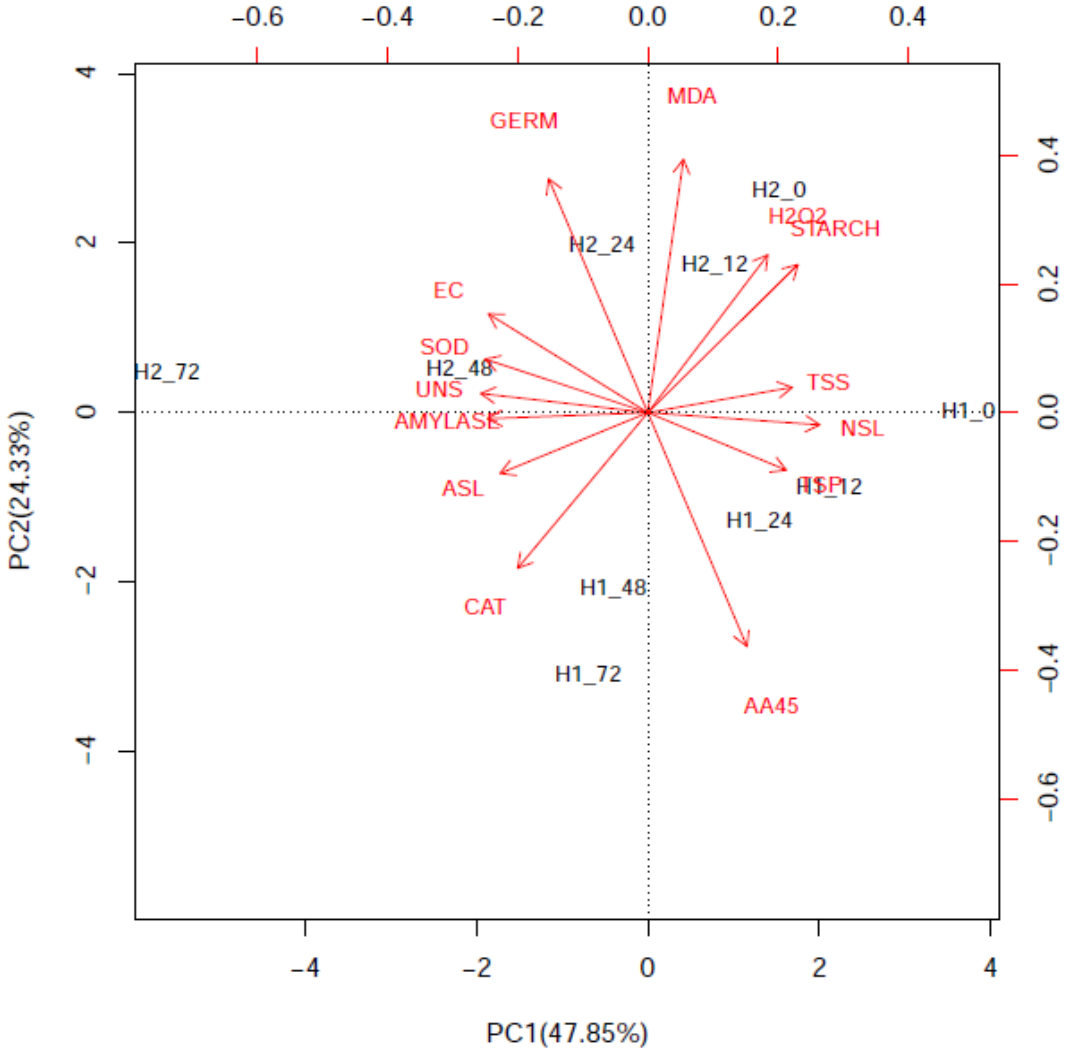


GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019.

Analysing the principal components diagram of the endosperm, it was observed that PC1 and PC2 were responsible for the capture of 47.85% and 24.33%, respectively, totalling

72.18% of the total data variation (Figure 10). As in the embryo, there was a clear separation between the H1 and H2 samples. The samples of H1 in the period of 0, 12 and 24 hours of stress were grouped in PC1+/PC2- by the variables of total soluble sugars, total soluble protein, percentage of normal seedlings and higher vigour by accelerated ageing. The samples of the H1 in the period of 48 and 72 hours were grouped in PC1-/PC2- by the variables of vigour by accelerated ageing and CAT. The H2 samples at 0 and 12 hours were pooled in PC1+/PC2 + by MDA, H_2O_2 and starch variables. Finally, the samples of H2 at the time of 24, 48 and 72 hours were grouped by the variables of electrical conductivity, SOD and percentage of unviable seeds.

Figure 10- Principal Component Analysis (PCA) of maize hybrids endosperm subjected to accelerated ageing for 0, 12, 24, 48 and 72 hours.

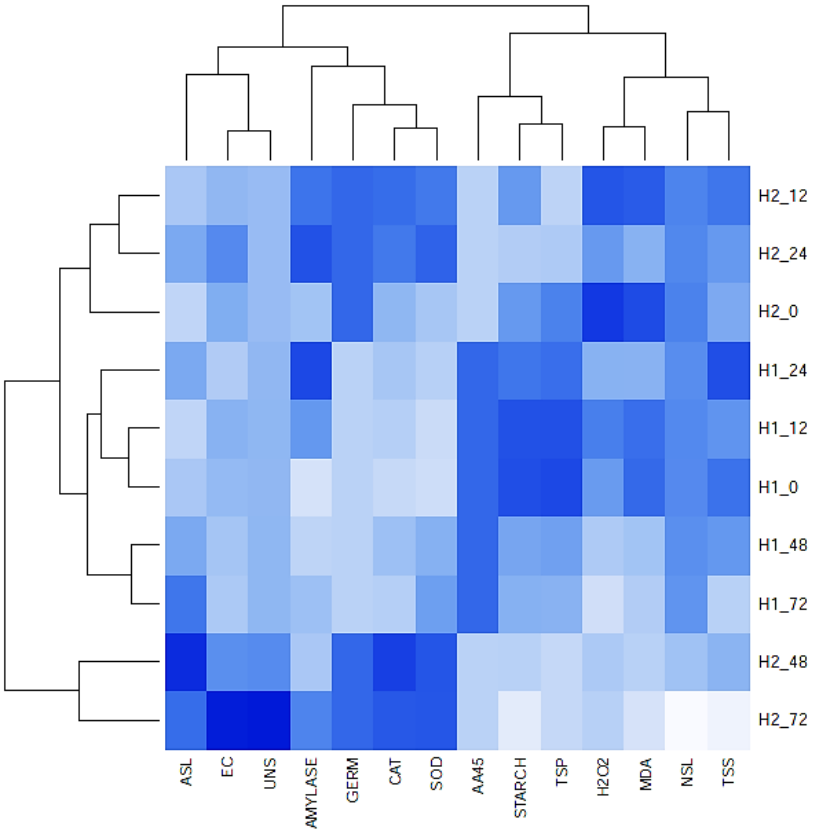


GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019

The cluster heat map was also applied to the physiological and biochemical parameters of the embryo and endosperm to identify the relationships between the evaluated variables. For the embryo, Figure 11 shows that the samples were grouped into 4 different groups. Group 1 was formed by H2 samples at periods of 0, 12 and 24 hours of stress. Group 2 was formed by the samples of H1 in periods of 0, 12 and 24 hours of stress. Group 3 was formed by samples of H1 in periods of 48 and 72 hours and finally, group 4 was formed by samples of H2 in the periods of 48 and 72 hours.

It is observed in Figure 11 that for the evaluated variables, the accelerated ageing was responsible for grouping the H1 embryo samples. The levels of starch, total soluble protein and total soluble sugars were observed with greater intensity in H1 samples. On the other hand, the variables SOD, CAT, MDA, H_2O_2, unviable seeds and abnormal seedlings were visualized with greater intensity in H2 samples.

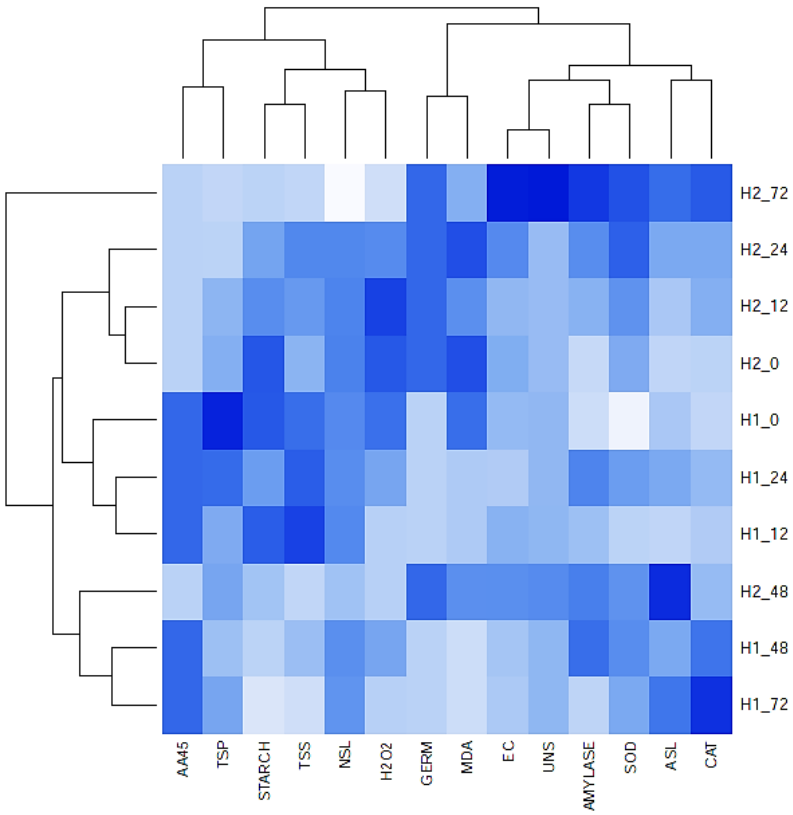
Figure 11- Hierarchical Cluster Analysis – Heat map (HCA) of embryo of maize hybrids subjected to accelerated ageing for 0, 12, 24, 48 and 72 hours.



GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019.

For the endosperm, Figure 12 shows that the samples were grouped into 5 different groups. Group 1 was formed by H2 samples at 72 hours of stress. Group 2 was formed by H2 samples at periods of 0, 12 and 24 hours of stress. Group 3 was formed by samples of H1 in periods of 0, 12 and 24 hours. Group 4 was formed by H2 samples in the 48 hour period. Finally, group 5 was formed by samples of H1 in the periods of 48 and 72 hours. Again, the H1 samples were grouped by vigour by accelerated ageing, total soluble protein content, starch and total soluble sugar.

Figure 12 - Hierarchical Cluster Analysis – Heat map (HCA) of endosperm of maize hybrids subjected to accelerated ageing for 0, 12, 24, 48 and 72 hours.

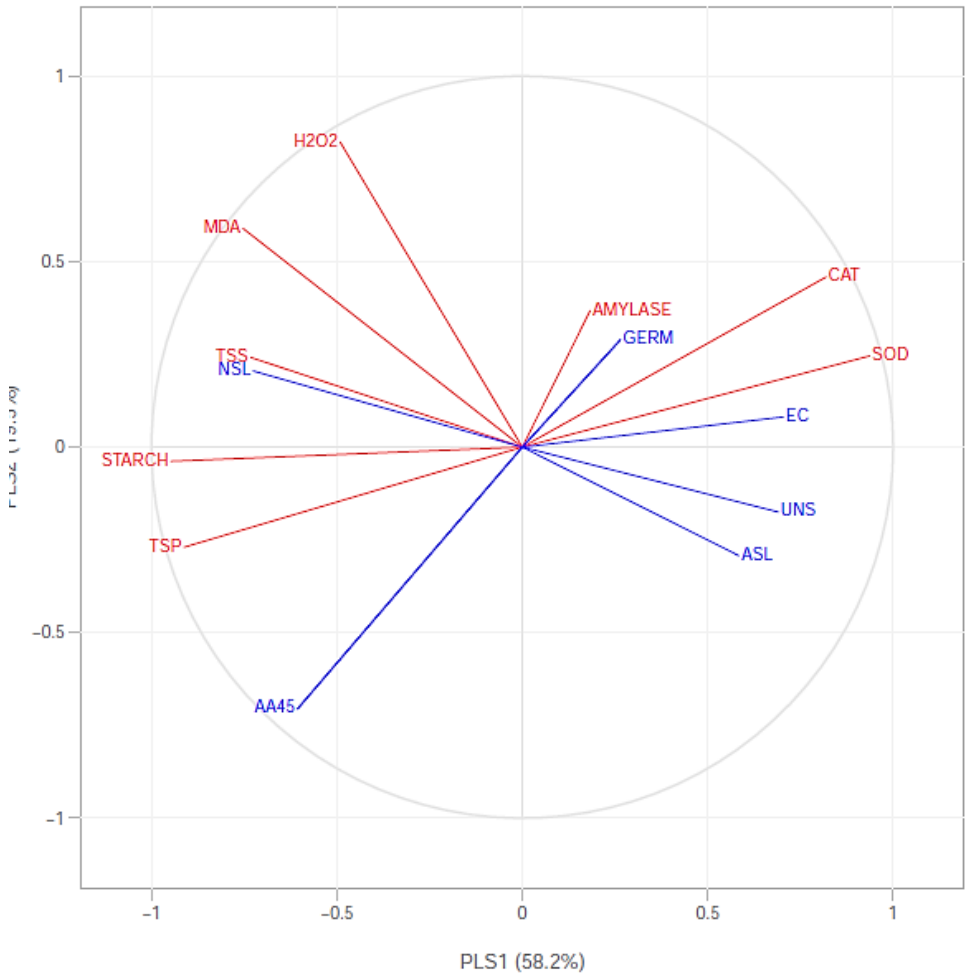


GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019.

The last statistical analysis applied to the data of this experiment was the Partial Regression of the Minimum Squares (PLS-R) to verify the existence of association between the physiological and biochemical variables of the embryo and endosperm. Figure 13 confirms that there was an association between the physiological quality by the accelerated ageing tests and percentage of normal seedlings with the biochemical components of total soluble protein, starch and total soluble sugars in the embryo. There was also the relationship between the electrical

conductivity with the higher SOD and CAT enzyme activity and with the percentage of unviable seeds.

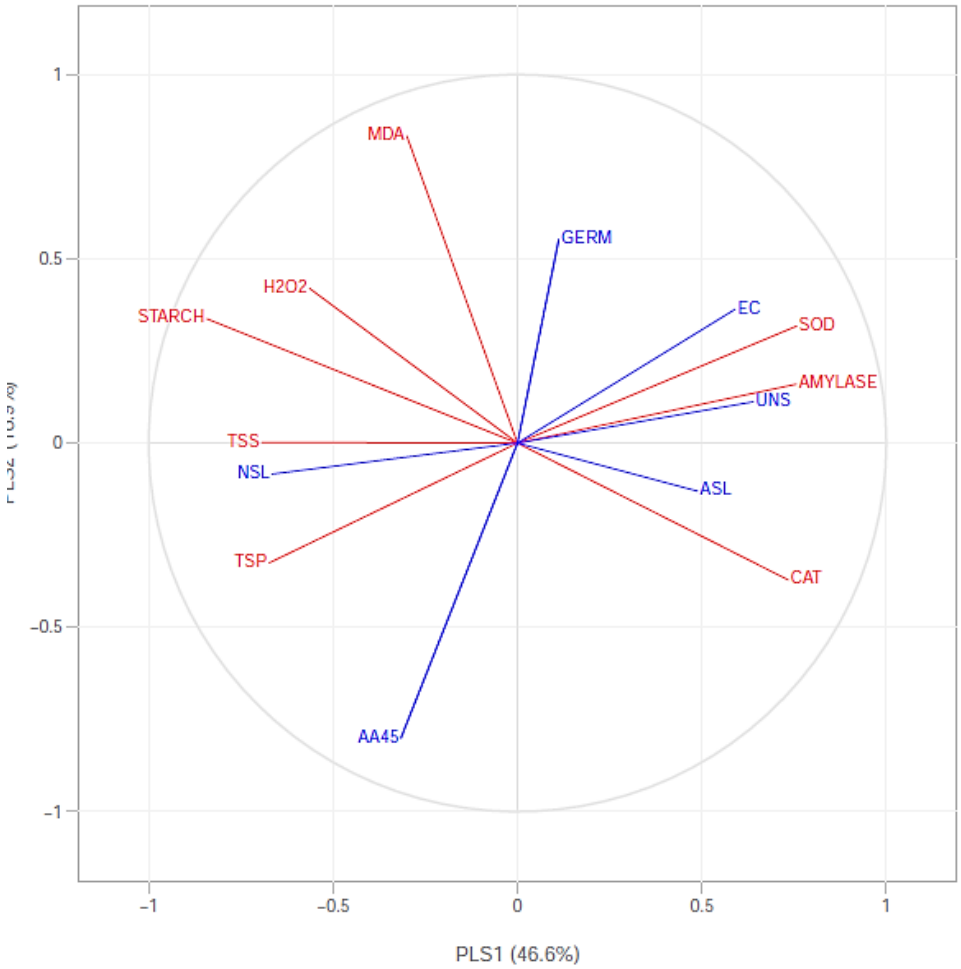
Figure 13 - Partial Least Square – Regression (PLS-R) of embryo of maize hybrids subjected to accelerated ageing for 0, 12, 24, 48 and 72 hours.



GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019.

Figure 14 shows the PLS-R of the endosperm, where it was again possible to observe the relationship between vigour by accelerated ageing and the percentage of normal seedlings with total soluble sugars, total soluble protein.

Figure 14 - Partial Least Square – Regression (PLS-R) of embryo of maize hybrids subjected to accelerated ageing for 0, 12, 24, 48 and 72 hours.



GERM – Germination; AA – Accelerated Ageing 45°C/72 hours; EC – Electrical Conductivity; NSL – Normal Seedlings; ASL – Abnormal Seedlings; UNS – Unviable Seeds, STARCH – Starch content; TSS – Total Soluble Sugar; AMYLASE – α-amylase activity; TSP – Total Soluble Protein; CAT – Catalase activity; SOD – Superoxide Dismutase activity; H_2O_2 – Hydrogen Peroxide; MDA – Malondialdehyde content.
Source: Elaborated by the author, 2019.

4.5 CONCLUSION

Hybrid maize seeds tolerant to accelerated ageing stress have the ability to maintain membranes integrity and to form normal seedlings after stress. This tolerance is dependent on the higher content of starch, soluble proteins and soluble sugars in the endosperm and the embryo.

Evaluating total the electrical conductivity, total soluble sugars, starch and total soluble protein contents provides early information on the physiological quality of seeds and can be used to select seeds with better physiological quality.

The analyses of PCA, HCA and PLS-R were efficient tools to show the degree of association amongst total soluble sugars, starch and total soluble protein content with the tolerance to accelerated ageing and to the formation of normal seedlings.

5 CHAPTER 3 - MODELLING THE VIGOUR OF MAIZE SEEDS SUBMITTED TO ARTIFICIAL ACCELERATED AGEING BASED ON ATR-FTIR DATA AND CHEMOMETRIC TOOLS

5.1 ABSTRACT

The first integrated metabolomic analysis of the embryo and endosperm of two contrasting maize hybrids in vigour level and subjected to accelerated ageing were performed using ATR-FTIR spectroscopy and chemometric analyses (PCA, HCA). The main goals of this research were to use ATR-FTIR spectroscopy associated with multivariate analyses to identify biochemical changes in high and low vigour seed tissues (embryo and endosperm) in response to accelerated ageing and to create a model to predict seed vigour based on spectroscopic data. High-vigour seeds undergo minimal changes in biochemical composition during stress by accelerated ageing while low-vigour seeds are more sensitive to stress and this lower tolerance is associated with reduced lipid and protein content and increased amino acids, carbohydrates and phosphorus compounds in the embryo. High-vigour seeds show an increase in peaks associated with amino acids and phosphorous compounds in the endosperm after 24 hours of stress while low-vigour seeds present these high-intensity peaks only after 72 hours in the embryo. The results prompts us to conclude that ATR-FTIR combined with chemometrics are powerful tools for screening the physiological quality of hybrid maize seeds and to predict the seed vigour of the samples and provides the theoretical basis for the genetic improvement of maize cultivars that aim at higher physiological seed quality.

5.2 INTRODUCTION

Maize (*Zea mays* L.) seeds carry all the genetic information of the crop and are essential to different purposes, such as for crop production and improvement, agricultural biotechnology, human nutrition, and food security. Seeds can be considered a key element in crop success, although it is dependent on a complex property called vigour (FINCH-SAVAGE; BASSEL, 2015). Seed quality, defined by improved vigour, is an essential trait, particularly during the current scenario of increasing uncertainty in food production due to climate change and the challenge of population growth expected by 2050 (HAMPTON et al., 2016). This characteristic, along with other characteristics of seed quality, is a determining factor for the germination and the establishment of crops in a fast and uniform way, in diverse environmental conditions (RAJJOU et al., 2012; MARCOS-FILHO, 2015; FINCH-SAVAGE; BASSEL, 2015, WEN et al., 2018).

The accelerated ageing test is considered one of the most sensitive tests for vigour assessment and consists of keeping the seed under high temperatures and high humidity for a fixed period (MARCOS-FILHO, 1999; BARRETO; GARCIA, 2017). Artificial accelerated ageing might cause accumulation of metabolic defects in seeds (GUTIERREZ et al., 1993) in different proportions than that in natural ageing. This methodology forms the basis of International Seed Testing Association (ISTA)-validated tests used in commercial seed testing for specific species. Thus, ageing is a key characteristic that is both a cause of differences in vigour and a basis for vigour testing (FINCH-SAVAGE; BASSEL, 2015). For such claimed reasons the accelerated ageing method was used in this study to model seed vigour test and to understand what biochemical changes occur in seed tissues in response to artificial ageing.

The increase in studies for the understanding of vigour has stimulated the investigation of biochemical components and their function in the physiological quality of seeds, since they can be used by biotechnology through the manipulation and enrichment of the composition of the tissues (YAN et al., 2014). Seed storage components such as proteins, lipids and carbohydrates, which are the main reserves, are synthesized and stored in the seed tissues during the maturation when they are still in the plant (COELHO; BENEDITO, 2008; BEWLEY et al., 2013; BAREKE, 2018, ZHAO et al., 2018). These storage components are involved in the germination and formation of seedlings providing carbon and nitrogen and, consequently, directly related to vigour (RAJJOU et al., 2012; BEWLEY et al., 2013; YAN et al., 2014; PRAZERES; COELHO, 2016; WU et al., 2017, NERLING et al., 2018). However, the research still lacks clarity as to which biochemical component is most affected by deterioration and,

consequently, by reduced vigour and where these alterations are occurring in the seed under stress conditions.

On the other hand, advances in high-throughput techniques of "omics" sciences have progressively lowered the barrier to accessing omics data (RAJJOU et al., 2012; BUESCHER; DRIGGERS, 2016; WU et al., 2017). Omics approaches, such as Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy (ATR-FTIR) generate data to provide biological insight based on statistical inference from datasets that are typically large. Multivariate statistical analysis (PCA, HCA, amongst others) contribute to reduce the dimensionality of data, to extract important information from the entire spectrum, improving reliability of the analyses and facilitating the interpretation of the results (KUHNEN et al., 2010). These data may be useful as seed vigour markers and provide information on which biological pathways may be related to the manifestation of physiological quality (VENTURA et al., 2012, RAJJOU et al., 2012) and are useful tools for investigating changes in the chemical composition of biological materials in response to stresses (KUMAR et al., 2016).

In-depth analyses of the mechanisms involved at the biochemical level have been considered efficient and important tools in many areas of research (ZHANG et al., 2012; OLIVEIRA et al., 2016; UARROTA et al. 2018). In seed physiology, there are no reports on the integration of ATR-FTIR with chemometrics to better understand the mechanisms related to seed vigour. In addition, the metabolomic profile of contrasting maize seeds subjected to accelerated ageing conditions remains unknown. In recent years, the mechanisms involved in the manifestation of seed vigour have been extensively studied in different species by many researchers around the world by other methods (CORBINEAU, 2012; VENTURA et al., 2012; MARCOS-FILHO, 2015; PRAZERES; COELHO, 2016; WU et al., 2017; NERLING et al., 2018; Gu et al., 2019).

However, the causes that determine this manifestation have not yet been fully elucidated by the research. It is fundamental to understand what determines the expression of seed vigour to improve it and to enhance the establishment of crops, given the great importance of this factor, since it is one of the main factors that are inextricably linked to the success or failure of the future harvest (MARCOS-FILHO, 2015, FINCH-SAVAGE; BASSEL, 2015). In addition, it is essential understand the mechanisms involved in seed deterioration to avoid losses of physiological quality, increasing the longevity of the seeds (SURESH et al., 2019).

In light of the above considerations regarding the contributions of the vigour study to seeds, in this research we tested two hypotheses: (i) there are differences in the biochemical composition of the embryo and endosperm of maize seeds and these differences are related to

seed vigour; (ii) the integration of ATR-FTIR profile datasets with chemometric techniques is a powerful tool for modelling biochemical markers related to seed vigour in maize. Based on these hypotheses, the first integrated metabolomic analyses of the embryo and endosperm of two contrasting maize hybrids at vigour level and subjected to accelerated ageing were performed using ATR-FTIR spectroscopy and chemometric analyses (PCA, HCA). The main objectives of this research were to use spectroscopy associated with multivariate analyses to identify biochemical changes in high and low vigour seeds tissues (embryo and endosperm) in response to accelerated ageing and to create a model for evaluating vigour through techniques based on these changes during stress.

5.3 MATERIAL AND METHODS

Two maize genotypes contrasting on vigour level (See Figure 1 for preliminary assay of genotype selection) were previously selected and used for this study using the germination rate (8 replicates of 50 seeds) and accelerated ageing test (4 replicates of 50 seeds). After that, seeds were submitted to accelerated ageing and then embryo and endosperm separated as described below.

Samples of maize seeds were distributed in a single layer on an aluminium screen and placed in gerbox boxes containing 40 mL of distilled water. The boxes were closed and placed in an ageing chamber for 12, 24, 48 and 72 hours at 45 °C. After each period, the samples were removed from the chamber and the seeds were frozen by liquid nitrogen. In addition, samples without stress condition (0 hours - control sample) were also frozen. All the seed samples had the embryo (embryonic axis and scutellum) separated from the endosperm, which were frozen by liquid nitrogen, ground using a grinder and stored at -20 °C until the infrared spectroscopy analysis.

The ATR – FTIR spectroscopy analysis was made in the embryo and endosperm samples from each maize genotype (H1 and H2) at each stress point (0, 12, 24, 48 and 72 hours), separately. Four replicates were used to obtain the samples and then a pool was obtained from the four samples for each time per hybrid before separation of the embryo and endosperm for infrared analysis. ATR-FTIR spectra were recorded in a Bruker IFS-55 (Model Opus v. 5.0, Bruker Biospin, Germany) spectrometer with a DTGS detector equipped with a golden gate single reflection diamond attenuated total reflectance (ATR) accessory (45° incidence-angle).

A background spectrum of the clean crystal was acquired and samples (100 mg) were spread and measured directly after pressing them on the crystal. The spectra were recorded at

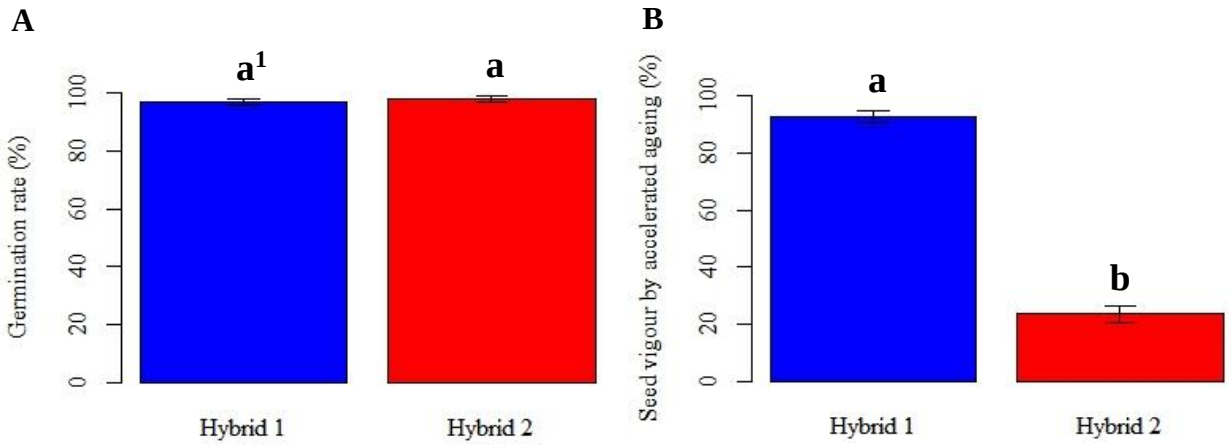
the transmittance mode from 400 to 4000 cm^{-1} (mid-infrared region) at the resolution of 4 cm^{-1}. Five replicates of the spectra were collected for each sample (20 samples) (UARROTA et al., 2013; UARROTA et al., 2014; UARROTA et al., 2017; UARROTA et al., 2018), totalling 100 spectra (2 hybrids – high and low vigour; 5 stress times - 0, 12, 24, 48 and 72 hours; 2 structures - embryo and endosperm x 5 replicates). Spectra were then normalised, baseline corrected and smoothed using Savitzky–Golay derivative function (SAVITZKY; GOLAY, 1964). Some regions were removed from both sides of all the spectra because of noise, making the region of interest from 600 to 3200 cm^{-1}. All pre-processing steps were performed in R software (R CORE TEAM, 2019).

Data of ATR-FTIR spectra were collected, pre-processed as described above and submitted to multivariate statistical analysis (Principal Component Analysis – PCA, Hierarchical Cluster Analysis – HCA). All analyses were performed in R software (R CORE TEAM, 2019) using scripts produced by Laboratory of Seed Analysis group.

5.4 RESULTS AND DISCUSSION

Preliminary results on the physiological quality of the seeds used in the experiment were obtained. The data collected were germination rate and vigour by accelerated ageing test at 45 °C for 72 hours (Figure 15).

Figure 15 - (A) Percentages of germination; and (B) seed vigour by accelerated ageing for the two hybrids evaluated previously this experiment.



[1]Mean values followed by the same lowercase letter belong to the same Tukey test group at 5% probability ($p<0.05$).
Source: Elaborated by the author, 2019.

In relation to the germination rate, the observed behaviour between hybrids 1 and 2 was similar ($p<0.05$), with percentages of 97 and 98%, respectively (Figure 15A). On the other hand,

the hybrids presented an extremely contrasting value for initial vigour by accelerated ageing, with significant statistical differences by the Tukey test ($p<0.05$), with values of 93% for hybrid 1 and 24% of vigour for the hybrid 2 (Figure 15B).

In studies related to seed vigour, it is essential that the percentage of germination be similar amongst the materials to be compared, to ensure that the differences are only in vigour and not in the physiological quality as a whole, making comparisons feasible (SBRUSSI; ZUCARELLI, 2014; MARCOS-FILHO, 2015), based on knowledge that there are genetic diversity for the vigour of maize seeds (PRAZERES; COELHO, 2016).

Amongst the main objectives of seed vigour testing is the ability to predict and to select seed lots that have the best quality before processing, storing them or taking them to be sown in the field (MARCOS-FILHO, 2015; FINCH-SAVAGE; BASSEL, 2015). The basic quality assessment through the germination test and vigour by accelerated ageing is inevitably time-consuming, requiring more than one week to complete and, furthermore, may not always correlate well with emergence under field conditions over a range of environmental conditions. Therefore, it is desirable to have a simple, reliable, accurate, rapid to perform, physiologically informative and relatively inexpensive test of seed quality involving vigour.

In studies of biological materials by ATR-FTIR, there are two important regions for the evaluation of the spectra called *fingerprint* region and functional groups region (LI-CHAN, 2010; BAKER et al., 2014). These regions must be exploited because they generally contain a large number of bands that can overlap each other, causing a single wave number to be related to more than one type of chemical component. The infrared in the middle region (400 to 4000 cm^{-1}) provides the recognition of functional groups present in chemical compounds (LI-CHAN, 2010). It allows the identification of similarities and dissimilarities of the biochemical composition between samples, such as the presence of carbohydrates, proteins and peptides, lipids and fatty acids, nucleic acids, amongst others (SOCRATES, 2001; ČERNÁ et al., 2003; LOPES; FASCIO, 2004; SILVERSTEIN et al., 2005; SCHULZ; BARANSKA, 2007; KUHNEN et al., 2010; LÓPEZ-SÁNCHEZ et al., 2010; KUMAR et al., 2016).

All peaks detected in the following spectra were identified with the aid of other publications (SOCRATES, 2001; ČERNÁ et al., 2003; LOPES; FASCIO, 2004; SILVERSTEIN et al., 2005; SCHULZ; BARANSKA, 2007; KUHNEN et al., 2010; LÓPEZ-SÁNCHEZ et al., 2010; KUMAR et al., 2016) because of the high complexity of spectra interpretation and their relation to the functional groups present in biological samples.

Most spectra of embryo (Figure 16 and Figure 17) presented high peak intensities in the functional group region (1800-4000 cm^{-1}), with peaks at 2860 and 2930 cm^{-1} that are related to amino acids, fatty acids, lipids, proteins and peptides (Table 19). Visually by the intensity of the bands, there were no chemical changes in these compounds for the stress times of 0, 12, 24 and 48 hours for both hybrids, which means that the behaviour of these compounds over accelerated ageing stress at 45 °C was similar for the high and low vigour hybrids (Figure 16 and Figure 17).

The main and most important differences in the embryo spectra between the hybrids were observed in the region above 2300 cm^{-1} at 72 hours stress. The high-vigour hybrid (H1) did not show changes in the intensity of these bands during the entire stress period, including 72 hours (Figure 16 - from A to E), while the low-vigour hybrid (H2) showed an expressive reduction of peak intensity close to 2930 cm^{-1} (amino acids, fatty acids, lipids, proteins and peptides) (Figure 17E). In addition, it was observed the absence of peaks close to 2860 cm^{-1} that is also related to the same compounds and were present in the low-vigour embryo (H2) before, indicating the possible degradation due to stress for 72 hours and the lower tolerance to this condition demonstrated by the low-vigour hybrid (Figure 17 – from A to E). The behaviour of the spectral peaks was different between embryos of the hybrids for the time of 72 hours, because the response to ageing is dependent on the genotype and initial vigour (OLIVEIRA et al., 2013, NERLING et al., 2013, PRAZERES; COELHO, 2016).

The high temperature (45 °C) and 100% relative humidity used in artificial ageing in the laboratory may cause damages and decrease the compounds related to the wave number near 2930 cm^{-1} in less vigorous hybrids, which is related to fatty acids, lipids, proteins, peptides and amino acids (Table 19). The decrease in lipid and fatty acids contents are associated with lipid peroxidation and reduction of antioxidant enzymatic activity caused by accelerated ageing stress in oat, macaw palm and wheat seeds (XIA et al., 2015; BARRETO; GARCIA; 2017; TIAN et al., 2019). Lipid peroxidation leads to the formation of free radicals that accelerate the deterioration of cell membranes, proteins and reduction of enzymatic activity, culminating in the reduction of the viability of the seeds (TIAN et al., 2019). In addition, natural or artificial accelerated ageing causes reduction in DNA integrity and protein synthesis, and these changes are closely associated to the reduction of germination under these deterioration conditions (GUTIÉRREZ et al, 1993).

Furthermore, the embryo of the low-vigour hybrid showed an increase in the bands near to 2342 and 2360 cm^{-1}, which are related to amino acids and phosphorus compounds. Most phytic acid, which is the way phosphorus is stored in seeds, is concentrated in the

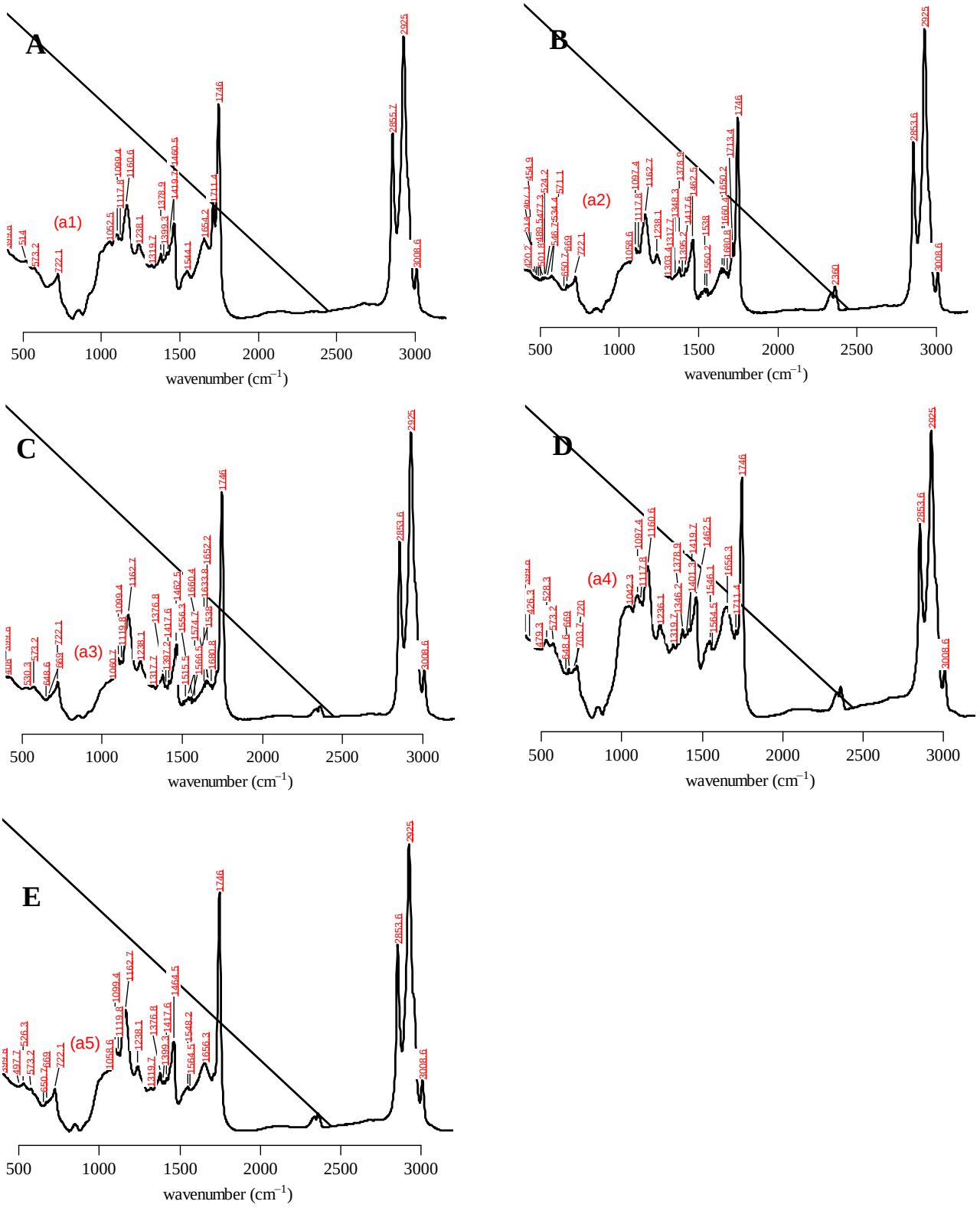
embryo (about 95%) and in aleurone layer in the endosperm (about 5%) of maize seeds, and its action as an antioxidant has been studied in this crop (LIN et al., 2005; DORIA et al., 2009; BEWLEY et al., 2013). In relation to the protein, most is found in the embryo (around 20%) while the endosperm content is close to 10% (CARVALHO; NAKAGAWA, 2012). Due to the observed changes in amino and phosphorus peaks after 72 hours in embryo samples, our results suggest the hypothesis that the lower vigour genotype undergoes greater degradation/hydrolysis of proteins and phytic acid, providing amino acids and phosphorus in the embryo in response to stress. Future studies will be carried out to investigate these relationships and the possibility of evaluating the content of phosphorus and amino acids after 72 hours of stress at 45 °C to separate vigour levels of hybrid maize seeds and the incorporation of these compounds to obtain seeds with higher physiological quality.

Many peaks, although with lower intensities were identified in the *fingerprint* region (400-1800 cm^{-1}) for both hybrids in embryo samples. Peaks in the region 900-1200 cm^{-1} are more correlated with the presence of carbohydrates in general (monosaccharides, oligosaccharides, polysaccharides), besides the presence of some amino acids, nucleic acids and phosphorus compounds. The presence of the band at 1260 cm^{-1} are related with amino acids, carbohydrates, nucleic acids, phosphate group, proteins and peptides (amide III). Other important bands identified in the embryo spectra of the hybrids were at 1650 and 1550 cm^{-1}, which are correlated to amino acids, nucleic acids, proteins and peptides (amide I and II). The last important band identified was at 1750 cm^{-1}, which is related to acetylated glycosides, amino acids, fatty acids, lipids, phospholipids, pectin, cellulose and nucleic acids (Table 19). These peaks were found in all spectra from time 0 (no stress) without major changes until the 48 hours of stress period for the two hybrids. The absence of biochemical changes for both genotypes up to 48 hours confirms the relationship of the components of the seed with vigour, indicating that both genotypes present tolerance to stress until this period.

The highest changes in intensity and presence of peaks were observed for the embryo samples collected after 72 hours of stress by accelerated ageing for the low-vigour hybrid, indicating behaviour similar to the region of functional groups (1800-4000 cm^{-1}) in the region of *fingerprint* (400-1800 cm^{-1}). For the high-vigour hybrid (H1), the bands referring to the *fingerprint* region remain unchanged throughout the stress (Figure 16A to 16E). For the low-vigour hybrid (H2), there was an expressive increase in the intensity of the peaks near 1044 and 1075 cm^{-1}, related to soluble sugars in general, structural carbohydrates and components with phosphorus in its composition (Figure 17E). In this same hybrid, there was

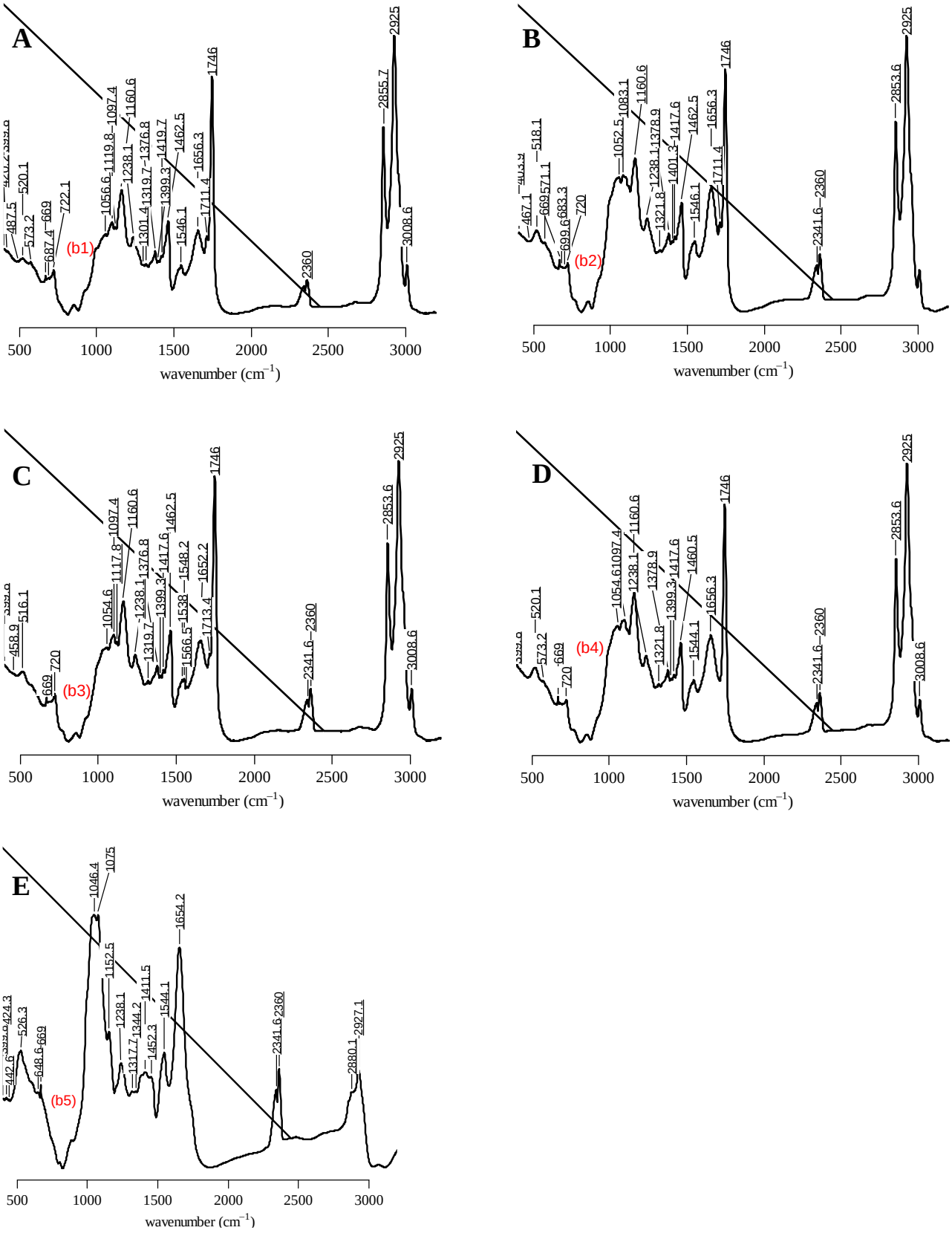
also an increase in intensity at the peak location near 1654 cm^{-1} (amino acids, nucleic acids and proteins), which was not present at the same intensity at other stress times. In addition, the visual analysis showed absence of the 1750 cm^{-1} peak (acetylated glycosides, amino acids, fatty acids, lipids, phospholipids, pectin, cellulose and nucleic acids) for the embryo of the low-vigour hybrid, indicating the possible degradation of these compounds and less tolerance to stress by the H2 (Figure 17E).

Figure 16- ATR-FTIR spectra of embryos of hybrid 1 (high vigour) during stress time by accelerated ageing. A – without stress (T0); B – 12 hours of stress (T12); C – 24 hours of stress (T24), D – 48 hours of stress (T48) and E – 72 hours of stress (T72).



Source: Elaborated by the author, 2019.

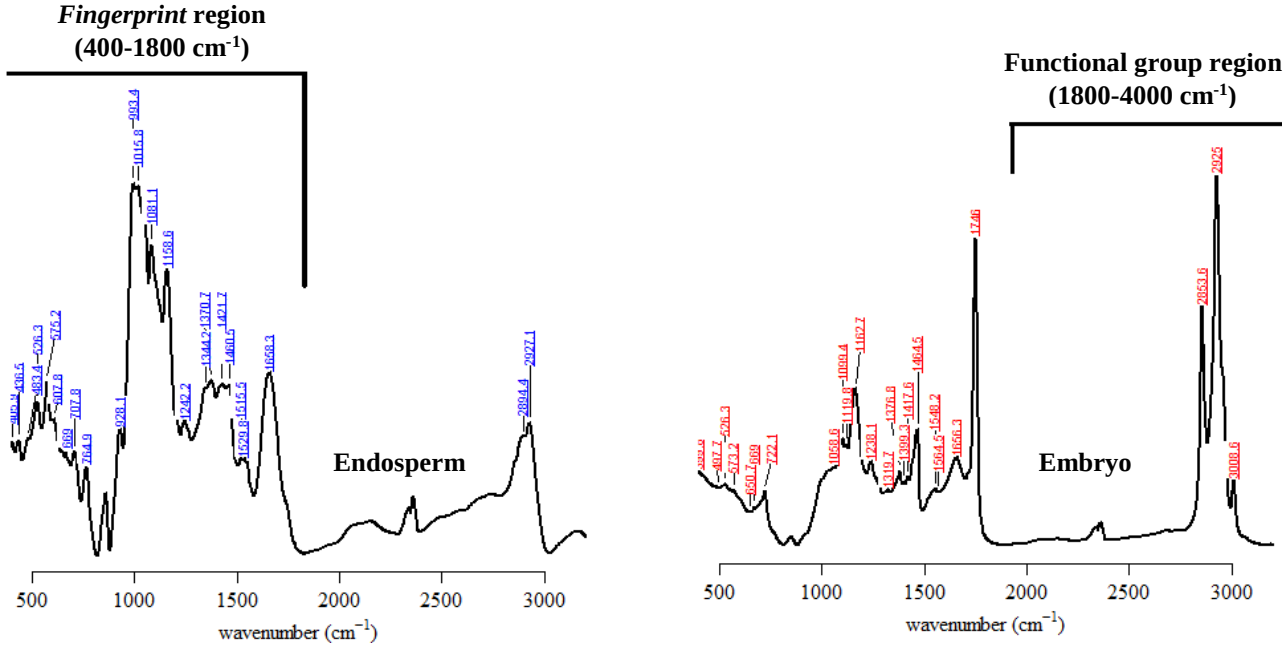
Figure 17- ATR-FTIR spectra of embryos of hybrid 2 (low-vigour) during stress time by accelerated ageing. A – without stress (T0); B – 12 hours of stress (T12); C – 24 hours of stress (T24), D – 48 hours of stress (T48) and E – 72 hours of stress (T72).



Source: Elbaorated by the author, 2019.

Contrarily to those previously observed in embryo, which show higher peak intensity in the region of the functional groups, the spectra of endosperm samples showed most peaks with high intensities in the fingerprint region (Figure 18). This behaviour is directly associated with a higher carbohydrate presence of the composition in the maize seed endosperm tissues, which are identified by the presence of bands in the fingerprint region, especially between 900 and 1200 cm^{-1} (SOCRATES, 2001; ČERNÁ et al., 2003; LOPES; FASCIO, 2004; SILVERSTEIN et al., 2005; SCHULZ; BARANSKA, 2007; KUHNEN et al., 2010; LÓPEZ-SÁNCHEZ et al., 2010; KUMAR et al., 2016).

Figure 18 - Characteristic profile of the ATR-FTIR spectra of the endosperm (left) and the embryo (right) of hybrid maize seeds.



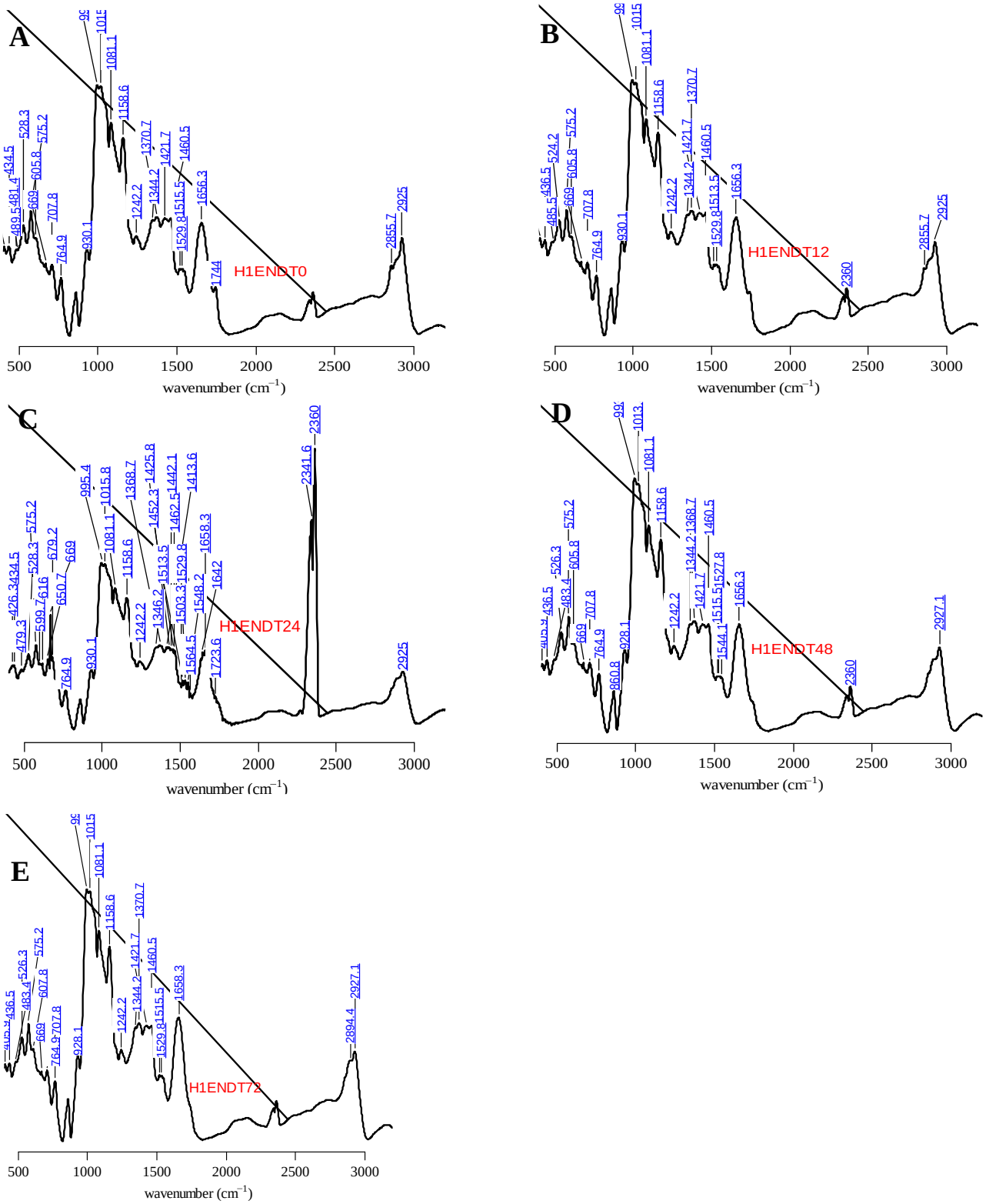
Source: Elaborated by the author, 2019.

The Figures 19 and 20 summarise the results of endosperm spectra for the two hybrids. The main differences between the endosperm spectra of high and low vigour were observed at 2342 and 2360 cm^{-1}, which are related to amino acids and phosphorus compounds. In all endosperm spectra of the hybrid of high vigour these peaks are present, drawing attention to the increase of intensity after 24 hours of stress, whereas in the hybrid of low vigour, these peaks are absent in all periods of stress in the endosperm.

Through the visual inspection of the spectra, it was observed that the embryo spectra presented higher number of peaks during stress when compared to the endosperm spectra, which means that this morphological structure presents the more severe symptoms of

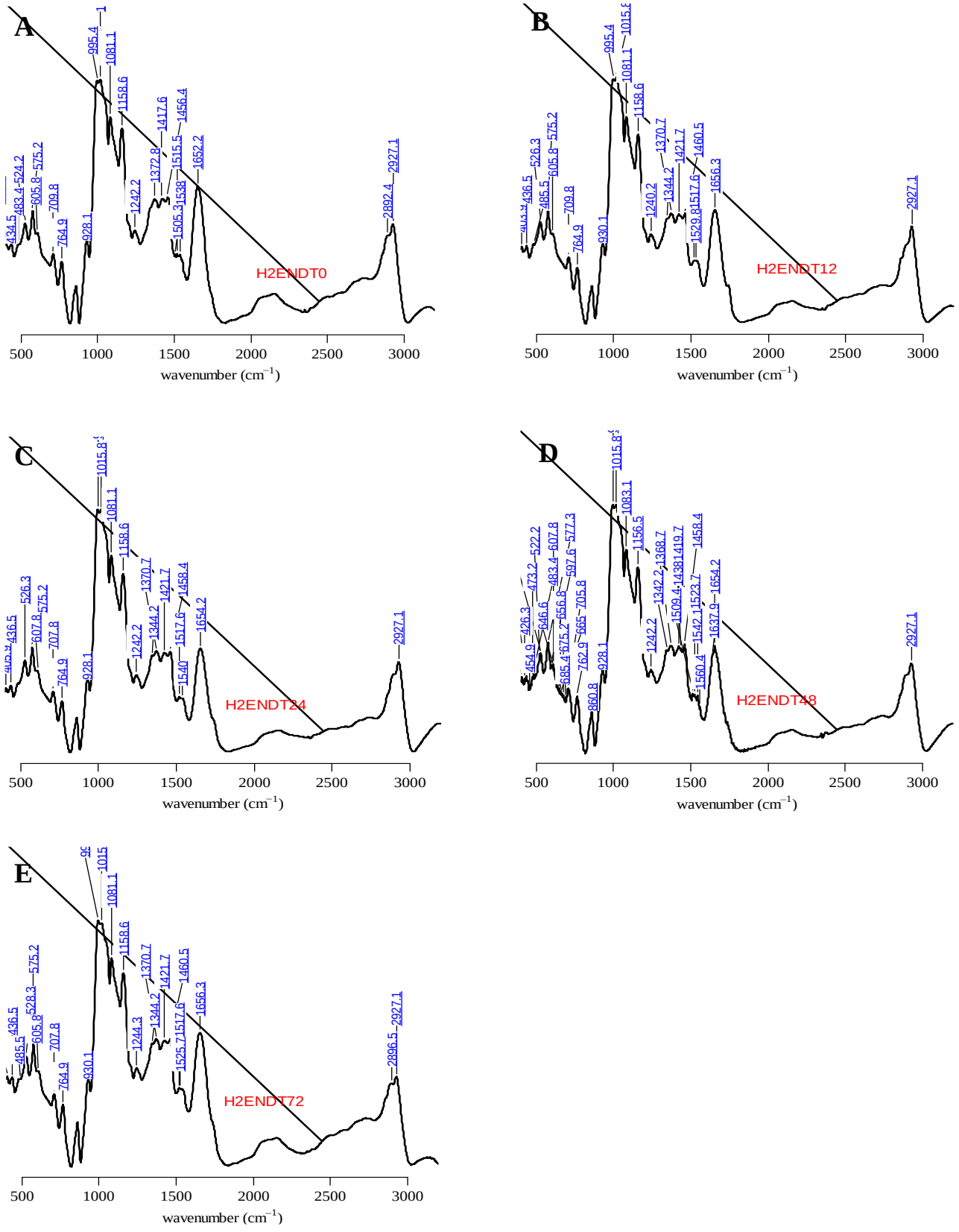
accelerated ageing. Similar results were found by HAN et al. (2017) in wheat seeds, where the metabolic changes found in the embryo were superior to those of the endosperm.

Figure 19 - ATR-FTIR of endosperm samples of hybrid 1 (high vigour) during stress time by accelerated ageing. A – without stress (T0); B – 12 hours of stress (T12); C – 24 hours of stress (T24), D – 48 hours of stress (T48) and E – 72 hours of stress (T72).



Source: Elaborated by the author, 2019.

Figure 20 - ATR-FTIR of endosperm samples of hybrid 2 (low vigour) during stress time by accelerated ageing. A – without stress (T0); B – 12 hours of stress (T12); C – 24 hours of stress (T24), D – 48 hours of stress (T48) and E – 72 hours of stress (T72).

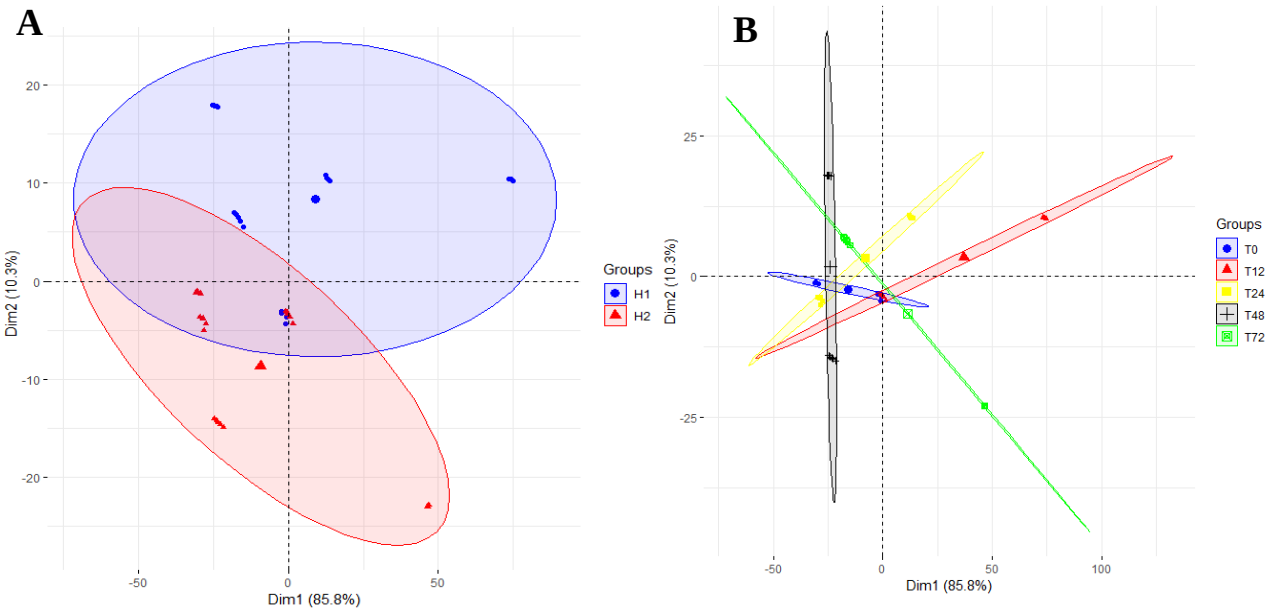


Source: Elaborated by the author, 2019.

The PCA was applied to all spectra (600-3200 cm^{-1}) and to selected peaks (regions without peaks were removed) in order to better understand the data patterns, to find clusters of samples and spectral peaks by directing such similarities or differences in the data. HCA and seriated heatmaps were also applied as a complementary analysis of PCA to uncover the factors that boosted the seed vigour. When PCA was applied to the embryo spectra considering the hybrids as a factor, the total variance captured was 96.1%, being 85.8% and 10.3% for PC1 and PC2, respectively (Figure 21A). Although one sample for the H1 hybrid (high vigour) and one sample for the H2 hybrid (low vigour) displaced from the other samples of the same hybrid, there was an effective separation between the two contrasting maize hybrids regarding the level of vigour (Figure 21A).

This separation allows us to assume that there are differences between the seed embryos spectra of high and low vigour, regardless of the stress time. Taking the stress time as factor in the same spectra it was not possible to separate the embryo samples meaning that the changes in spectra during the stress time were minimal (Figure 21B).

Figure 21 - (A) PCA of all spectra region (600-3200 cm^{-1}) of embryo samples taking the two hybrids as a factor. (B) PCA of all spectra region of embryo samples taking the stress time as factor.

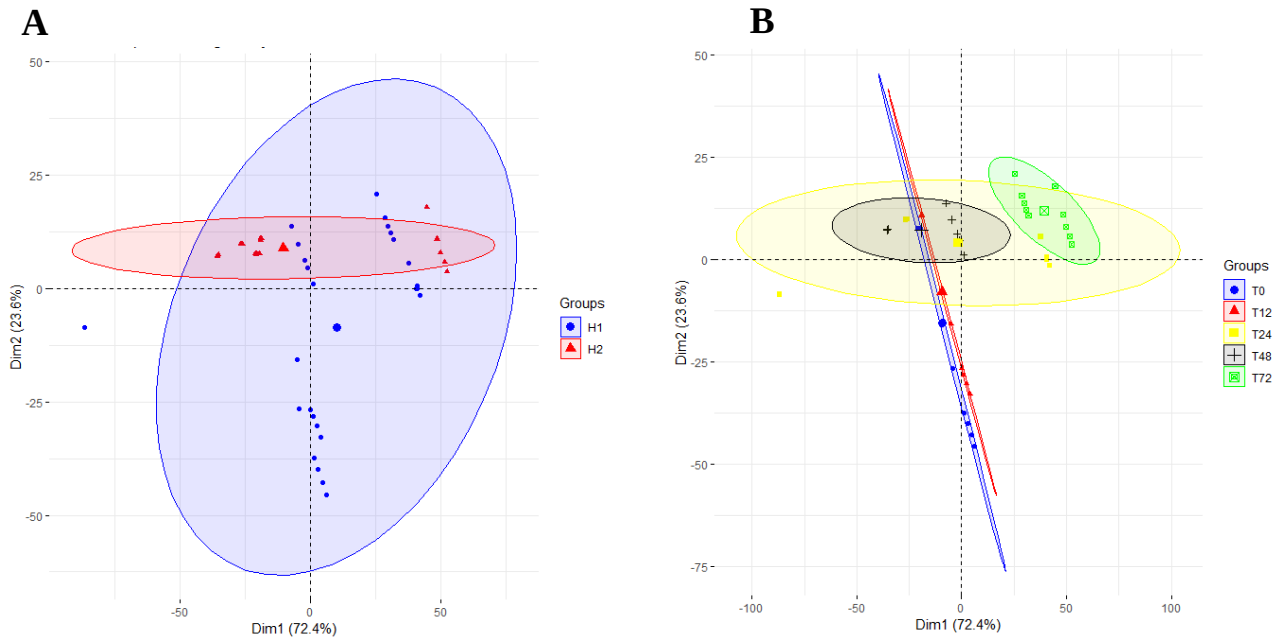


Source: Elaborated by the author, 2019.

Regarding the maize endosperm samples (Figure 22A), the total variance captured by PCA taking the maize hybrids as factors was 96%, being 72.4% and 23.6% the variances for PC1 and PC2, respectively. Taking the stress time as factor, there was a separation between

samples, except for the samples of 24 and 48 hours of stress of the two hybrids and those stressed during 72 hours clustered together independently of the hybrid (Figure 22B). Such results indicate that there are structural changes starting from 24 hours of stress.

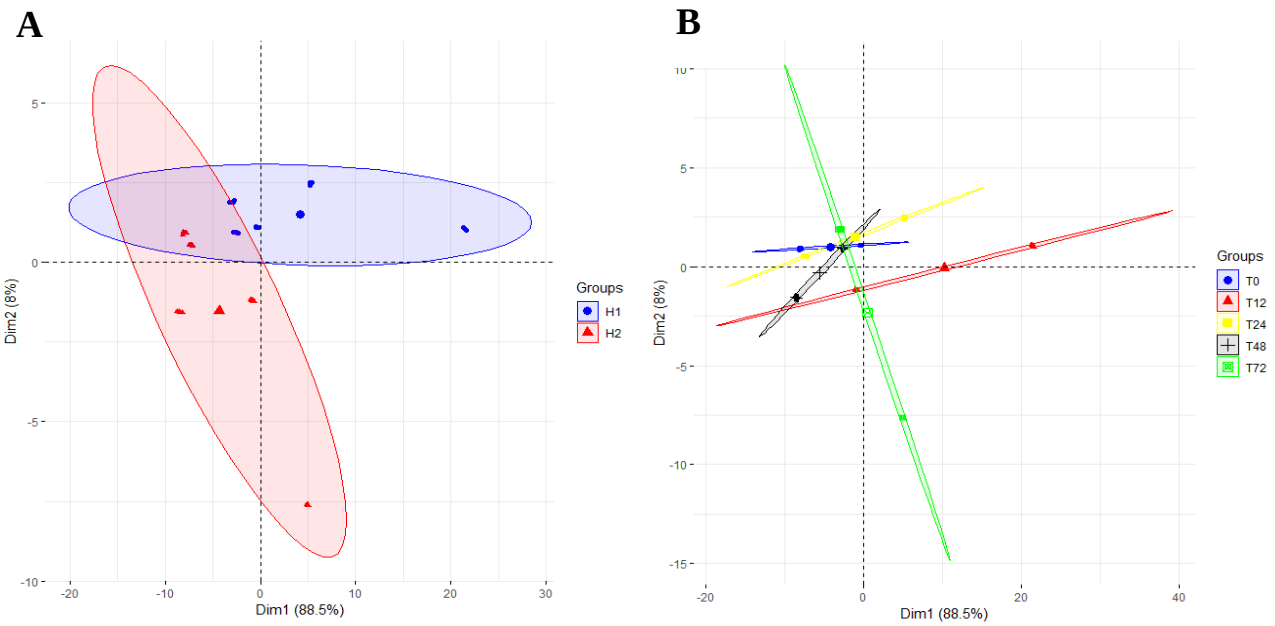
Figure 22- (A) PCA of all spectra region (600-3200 cm^{-1}) of endosperm samples taking the two hybrids as a factor. (B)PCA of all spectra region of endosperm samples taking the stress time as factor.



Source: Elaborated by the author, 2019.

PCA analysis of the selected peaks of embryo spectra was also done (Figure 23). The total variance captured was 96.5%, being 88.5% and 8% for PC1 and PC2, respectively. Despite the small improvement in the variance, there was no improvement in the grouping of samples when doing the analysis only with the selected peaks when compared to the analysis of the total region of the spectra. Differences were observed only between hybrids (Figure 23A) and not between times of stress (Figure 23B).

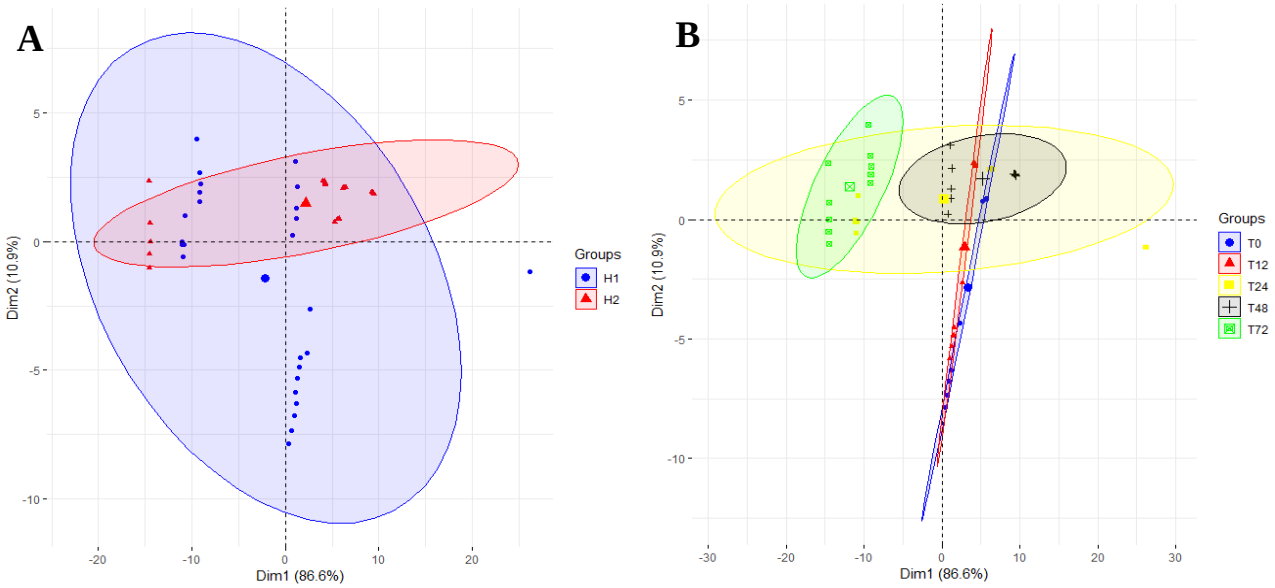
Figure 23 - (A)PCA of selected peaks of embryo samples taking the two hybrids as a factor. (B) PCA of selected peaks of embryo samples taking the stress time as factor.



Source: Elaborated by the author, 2019.

PCA analysis of the selected peaks of endosperm spectra showed 97.5% of total variance captured (Figure 24A), being 86.6% and 10.9% for PC1 and PC2, respectively. Differences were observed between hybrids and time of stress. Samples without stress and 12 hours of stress were similar. This tendency was also observed for the stressed samples for 24 and 48 hours, while those samples stressed for 72 hours were totally different from the others (Figure 24B).

Figure 24 - (A) PCA of selected peaks of endosperm samples taking the two hybrids as a factor. (B) PCA of selected peaks of endosperm samples taking the stress time as factor.

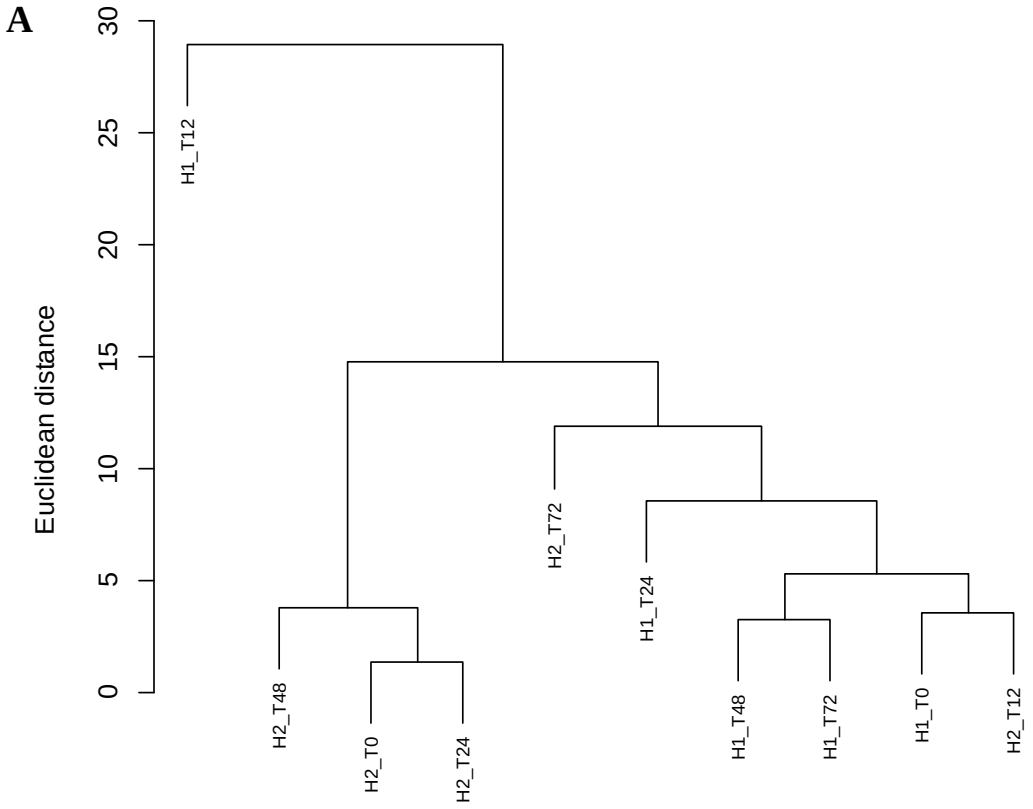


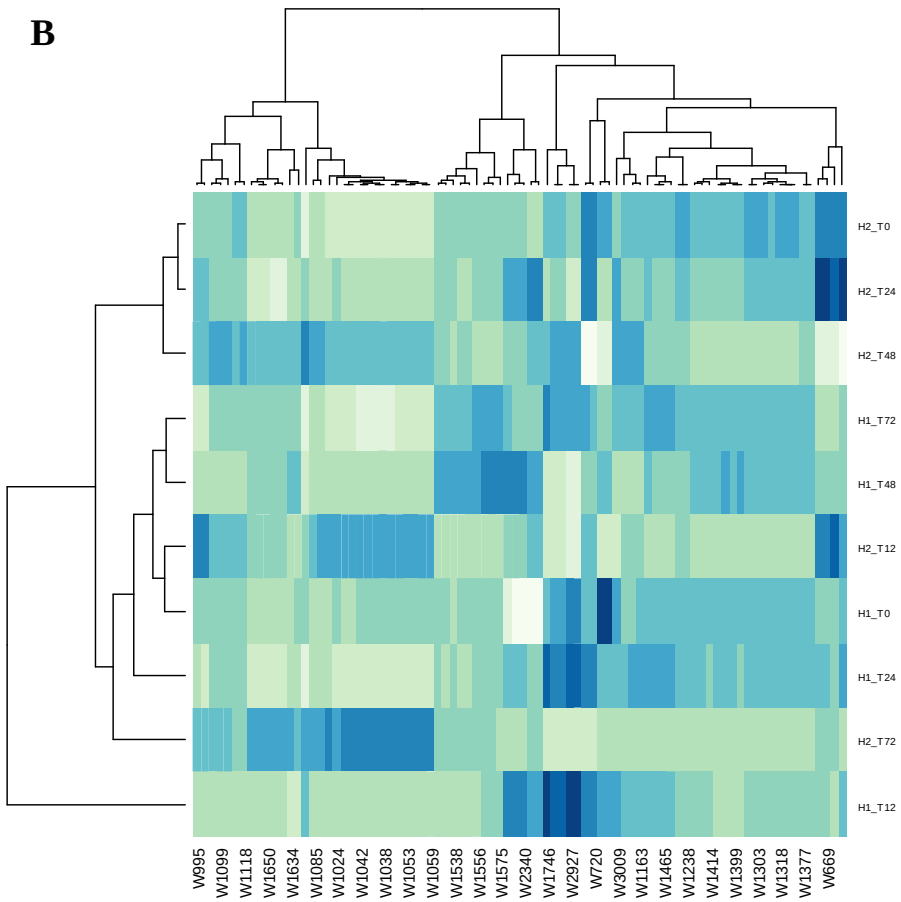
Source: Elaborated by the author, 2019.

When hierarchical cluster analysis and seriated heatmaps were applied to the selected spectral peaks for embryo and endosperm, better insights were found. As it can be observed in the Figure 25, in relation to the embryo samples, three different groups can be observed. The first group was composed by H1_T12 (samples of hybrid 1 stressed during 12 hours), the second group by H2_T0, H2_T24 and H2_T48 hours; and the last group by H1_T0; H1_T24, H1_T48, H1_T72, H2_T12 and H2_T72 (Figure 25A).

The sample H2_72 showed to be in the last group, but a deep analysis of seriated heatmaps shows that the H2_72 samples have characteristic peak intensities at 1024, 1059, 1180, 1650 and 900-1099 cm^{-1} related to polysaccharides, proteins and carbohydrates. The sample H1_12 was separated alone mainly due to the presence of the peaks at 1750 and 2927 cm^{-1} that are related to acetylated glycosides, amino acids, fatty acids, lipids, phospholipids, proteins and peptides.

Figure 25 - (A) HCA of selected peaks of embryo samples; and (B) Heatmap of selected peaks of embryo samples.





Source: Elaborated by the author, 2019.

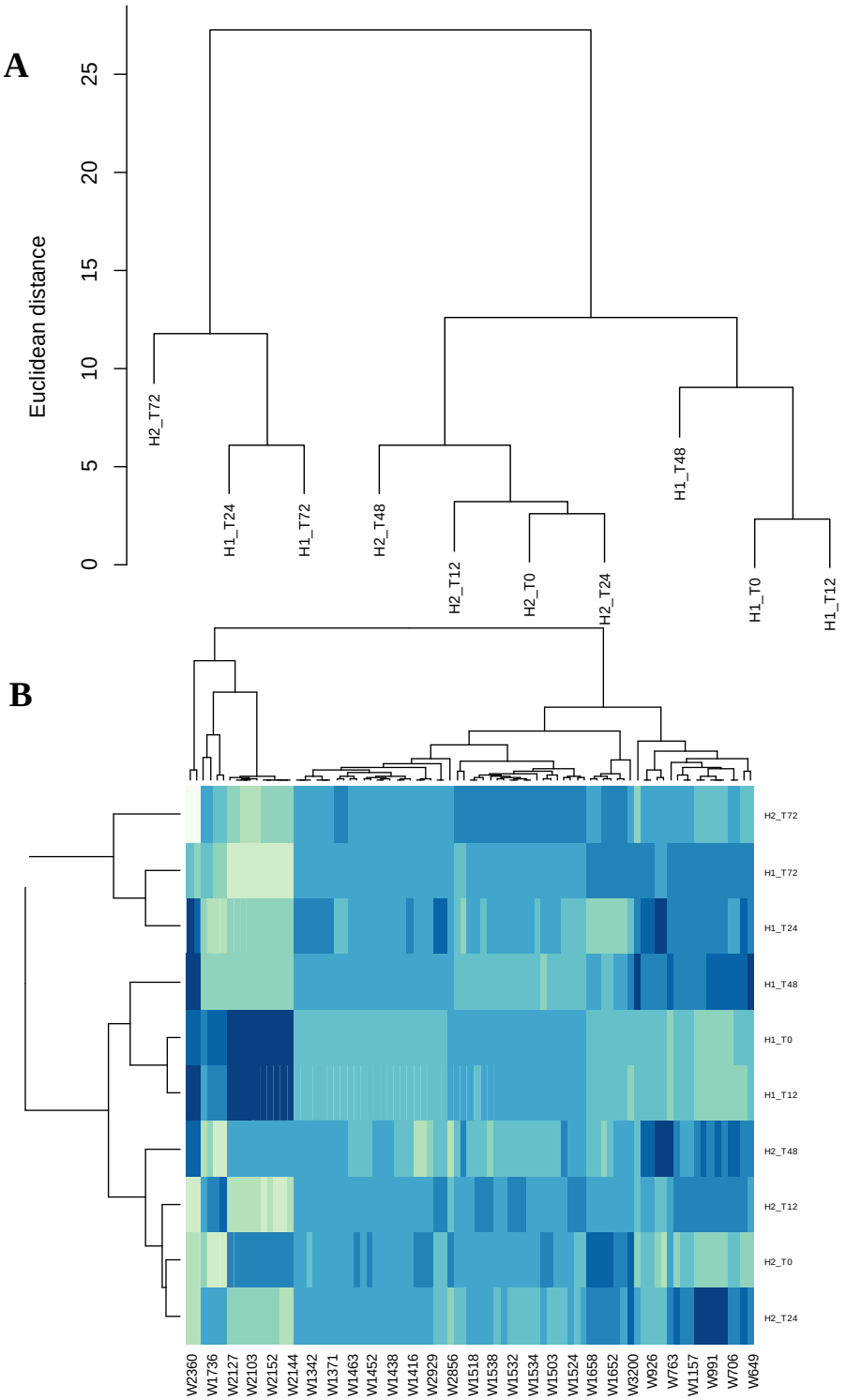
The figure 26 shows the HCA (Figure 12A) and serieted heatmaps (Figure 26B) of endosperm samples. The H2_T72 sample was grouped alone in the HCA, but close to the group composed of samples from hybrid 1 (H1_T24 and H1_T72), as it showed similarity of peaks with these samples (Figure 26A). The presence of similar peaks between H2_T72, H1_T72 and H1_T24 was observed in the serial heat maps (Figure 26B), especially those near 1370, 1421, 1460, 1515 e 1529 cm^{-1}, related to structural carbohydrates (cellulose, xyloglucan), nucleic acids, amino acids and proteins (amides I and II) from the endosperm (Table 19).

The main dissimilarity was found in the peak of 2342 and 2360 cm^{-1} present in H1_T24 and absent in the other samples. These peaks are associated to phosphorus compounds, which showed great intensity in this sample, indicating changes in this compound due to the stress period in this hybrid.

The second group in the HCA was composed by samples of hybrid 2, being H2_T0, H2_T12 and H2_T24 (Figure 26A). The H2_T48 samples were similar to this second group in the HCA due to the peaks associated to carbohydrates, but are distinct from the others due to greater changes in lipids, proteins, amino acids and nucleic acids in this sample, indicating that

in the stress period of 0, 12, 24 and 48 hours by accelerated aging did not cause major changes in the biochemical composition of the hybrid endosperm 2. The last group consisted of samples from hybrid 1, where H1_T0 and H1_T12 were also close to H1_T48 (Figure 26A), which were grouped mainly by carbohydrates and proteins (Figure 12B) (Table 19).

Figure 26- (A) HCA of selected peaks of endosperm samples; and (B) Heatmap of selected peaks of endosperm samples.



Source: Elaborated by the author, 2019.

Deep analyses showed that there was a small change in the components of the endosperm reserve during accelerated ageing stress for the two hybrids regardless of the level of vigour. The main change along the stress was observed in the samples of H1_T24, where there was an expressive increase in the intensity of the peaks of 2342 and 2360 cm^{-1}. This indicates that, at that time, the high-vigour hybrid required a greater amount of phosphorus compounds, probably to excel in stress and survive to form a normal plant, even under adverse conditions, which was not observed in the endosperm of low-vigour hybrid.

On the other hand, in the embryo spectra, the greatest differences were observed in the time of 72 hours for the low-vigour hybrid, where drastic reductions in the intensities of the peaks close to 2880 and 2927 cm^{-1} were observed, associated to a possible deterioration of lipids, fatty acids and proteins. In addition, there was an increase in the peaks associated with carbohydrates (mono, oligo and polysaccharides) and phosphorus compounds, possibly caused by the increased demand for these components in an attempt to overcome stress. In the high-vigour hybrid, there were no significant changes to the embryo spectra along the stress, meaning that this hybrid has greater stability in the compounds when subjected to adverse conditions of high temperatures (45 °C) and 100% relative humidity, regardless of the period of exposure.

Table 19 - Main compounds identified in the embryo and endosperm samples during the stress by accelerated ageing.

	Main compounds identified
1024	Carbohydrates: cellulose, hemicellulose, polysaccharides, starch, sucrose
1030	Carbohydrates: amylopectin, amylose, cellulose, galactose, hemicellulose, pectic polysaccharides, pyranose compounds, starch, sucrose
1044	Carbohydrates: amylopectin, amylose, cellulose, fructose, pectic polysaccharides, pyranose compounds, starch, sucrose, xyloglucan
1059	Carbohydrates: amylopectin, amylose, arabinose, cellulose, fructose, glucose, hemicellulose,
1057	pectic polysaccharides, pyranose compounds, starch, sucrose
1075	Carbohydrates: cellulose, hemicellulose, pectic polysaccharides, pyranose compounds, ribose, starch, sucrose, xyloglucan
	Phosphorus compounds
1099	Amino acids
	Carbohydrates: cellulose, galactose, hemicellulose, pectic polysaccharides, pyranose compounds, ribose, starch, sucrose
	Nucleic acids
	Phosphorus compounds
1120	Amino acids
	Carbohydrates: cellulose, hemicellulose, pectic polysaccharides, pyranose compounds, starch, sucrose
	Nucleic acids
1180	Amino acids
	Carbohydrates: cellulose, hemicellulose, pectic polysaccharides, pyranose compounds, starch, sucrose
	Nucleic acids
1260	Amino acids
	Carbohydrates: cellulose, hemicellulose, pectic polysaccharides, pyranose compounds
	Nucleic acids
	Phosphorus compounds
	Proteins and Peptides (amide III)
1379	Carbohydrates: cellulose, xyloglucan
	Lipids
	Nucleic acids
1399	Amino acids
	Nucleic acids
	Lipids
	Protein and peptides: polyglicynes
1550	Amino acids
	Nucleic acids
	Protein and peptides (amide II): polypeptides
1650-1654	Amino acids
	Nucleic acids
	Protein and peptides (amide I): polyglycines and polypeptides
1746-1750	Acetylated glycosides
	Amino acids
	Carbohydrates: pectin, cellulose
	Fatty acids, lipids, phospholipids
	Nucleic acids
2342-2360	Amino acids
	Phosphorus compounds
2854	Amino acids
	Fatty acids, lipids
	Proteins and peptides: polyglycines
2927-2960	Amino acids
	Fatty acids, lipids
	Proteins and peptides: polyglycines

Source: Socrates, 1994; Černá et al., 2003; Lopes and Fascio, 2004; Silverstein et al., 2005; Schulz and Baranska, 2007; Kuhnen et al., 2010; López-Sánchez et al., 2010; Kumar et al., 2016. Elaborated by the author, 2019.

5.5 CONCLUSIONS

The study prompt us to conclude that ATR-FTIR combined with chemometrics are powerful tools for screening the physiological quality of hybrid maize seeds and to predict the seed vigour of the samples and provides theoretical basis for the genetic improvement of maize cultivars that aim at higher physiological seed quality.

High-vigour seeds undergo minimal changes in biochemical composition during stress by accelerated ageing, evidencing the relation of the compounds with the vigour of the seeds. Low-vigour seeds are more sensitive to stress and this lower tolerance is associated with reduced lipid and protein content and increased amino acids, carbohydrates and phosphorus compounds in the embryo.

High-vigour seeds show increase in peaks associated with amino acids and phosphorous compounds in the endosperm after 24 hours of stress. Low-vigour seeds present these high intensity peaks only after 72 hours in the embryo.

6 CONSIDERAÇÕES FINAIS

O cultivo de milho híbrido representa uma parcela importante da produção de grãos no Brasil e no mundo, fazendo com que a produção e utilização de sementes com elevada qualidade seja essencial para obtenção de lavouras altamente produtivas. O potencial produtivo é dependente da uniformidade do estabelecimento de plântulas a campo e, consequentemente, da alta qualidade fisiológica de sementes. Assim, avaliar a tolerância de cultivares à determinados estresses traz benefícios econômicos a todo o sistema de produção.

No presente estudo, uma análise comparativa das respostas fisiológicas e bioquímicas de sementes de milho híbrido e suas relações com a manifestação do vigor foi conduzida em resposta ao envelhecimento acelerado. Foram verificadas distinções entre os mecanismos bioquímicos de sementes de alto e baixo vigor, principalmente em relação à proteína solúvel total, carboidratos como açúcares solúveis totais e amido. As sementes de alto vigor demonstraram maior estabilidade de membranas celulares e tolerância ao estresse, enquanto que sementes de baixo vigor foram sensíveis ao estresse e essa sensibilidade foi associada ao aumento do metabolismo na tentativa de superação da condição adversa imposta às sementes.

Assim, foi possível abrir novos caminhos para a pesquisa, sendo necessária a condução de novos estudos para descobrir quais são essas proteínas e carboidratos que estão envolvidos na expressão do vigor de sementes de milho híbrido para melhorar a tolerância da cultura a condições ambientais estressantes. A exemplo, pode-se citar a tolerância aos processos de envelhecimento acelerado e, consequentemente, do aumento do período de armazenamento sem que ocorram perdas severas de qualidade. Ou ainda, a utilização dos mecanismos compreendidos como base para o entendimento do vigor em respostas a outros tipos de estresse.

A grande importância do uso de ferramentas como as análises “ômicas" tais como proteômica, metabolômica, transcriptômica, entre outros, são os benefícios no entendimento de como e onde essas reações ocorrem em sementes de alto e baixo vigor, fundamentais para auxiliar a desvendar os processos, com o intuito de interferi-los por meio do melhoramento e da engenharia genética, visando a obtenção de sementes de maior qualidade. No entanto, faz-se essencial retornar os pensamentos para a base de toda a pesquisa da área de sementes, constituindo-se na principal “ômica” a qual esse estudo almeja atingir: a agronômica. Dessa forma, os resultados desse estudo contribuem para que a produção de alimentos continue crescendo e de forma sustentável, pois a pesquisa somente pode ser fundamentada se aplicada em benefício da população.

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