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PROGRAMA DE PÓS-GRADUAÇÃO EM AGRICULTURA E BIODIVERSIDADE

SEED TREATMENT, STRESS TOLERANCE, AND GENETIC VARIABILITY OF *Moringa oleifera* Lam.

TÁSSIA FERNANDA SANTOS NERI SOARES

2023

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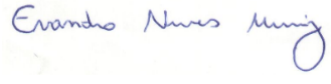

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SÃO CRISTÓVÃO
SERGIPE – BRASIL

Aos meus pais, Josenilda e José Soares e ao meu companheiro de vida, Vinícius Leite, pelo apoio incondicional
Dedico

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BIOGRAFIA

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AMOVA Analysis of Molecular Variance
APX Ascorbate peroxidase
AsA Ascorbic Acid
BA Benzyladenine
BR Brassinosteroids
CAT Catalase
CRD Completely randomized design
DAS Days after sowing
DNA Deoxyribonucleic Acid
DR Day recovery
DS Day stress
DW Dry weight
EBL Epibrassinolide
EDTA Ethylenediaminetetraacetic acid
FCG First Germination Count
F_v/F_m maximum quantum yield efficiency of PSII
FW Fresh weight
G Germination
He Expected heterozygosity
I Shannon index
ISSR Inter Simple Sequence Repeats
LA Leaf Area
LT_{50} Lethal temperature causing 50% injury
MDA Malondialdehyde
MGT Mean Germination Time
MLE Moringa Leaf Extract
NA Non-acclimated
Na Number of expected alleles
Ne Number of effective alleles
PAR Photosynthetically active radiation
PCoA Principal coordinate analysis
PCR Polymerase Chain Reaction
PSII Photosystem II
PVPP Polyvinylpolypyrrolidone
ROS Reactive Oxygen Species
RP Root Protrusion
RPSI Root Protrusion Speed Index
UFC Unfrozen control
UNS Unstressed
UPGMA Unweighted Pair Group Method with Arithmetic Means

RESUMO

SOARES, Tássia Fernanda Santos Neri. **Tratamento de sementes, tolerância a estresses e variabilidade genética em *Moringa oleifera* Lam.** São Cristóvão: UFS, 2023. 82p. (Tese – Doutorado em Agricultura e Biodiversidade) *

Moringa oleifera Lam. é uma espécie arbórea popularmente conhecida pelos seus múltiplos usos. Devido ao alto valor nutricional das suas folhas, a moringa pode ser utilizada tanto na alimentação humana quanto na alimentação animal. Nativa da Índia, essa espécie é amplamente difundida em regiões tropicais e subtropicais do mundo. Assim, esse estudo teve como objetivos: (1) discutir a importância do extrato foliar de moringa e verificar a sua eficiência no tratamento de sementes; (2) avaliar a tolerância dessa espécie ao estresse de baixas temperaturas; e (3) estimar a diversidade genética dos acessos do Banco Ativo de Germoplasma de Moringa da Embrapa Tabuleiros Costeiros. Para isso, o extrato foliar de moringa (MLE 1:30) juntamente com os demais tratamentos (condicionamento em água, 24-epibrassinolideo (EBL 10^{-10}, 10^{-8}, and 10^{-6} M) e o ácido ascórbico (AsA 50, 100, and 150 $mg.L^{-1}$)) foram utilizados para avaliar a germinação e crescimento de plântulas de moringa. Para determinar a tolerância a baixas temperaturas, plântulas de moringa foram submetidas às temperaturas de 20/15, 15/10 e 10/5 °C por 7 e 14 dias. Com isso, os efeitos da duração do estresse e da recuperação em condições ótimas foram avaliados por meio de parâmetros morfológicos e fisiológicos. Além disso, foi avaliado se essa espécie possui capacidade de tolerar temperaturas abaixo de zero por meio de um protocolo laboratorial de congelamento e descongelamento e se possui habilidade de aclimatação. A diversidade genética de 25 acessos compostos por 177 genótipos do BAG de Moringa foi estimada por meio do uso de 20 marcadores moleculares *ISSR*. Como resultados, observou-se que o condicionamento de sementes favoreceu a germinação e o crescimento de plântulas de moringa. Além disso, observou-se aumento nos parâmetros morfológicos e fisiológico nos tratamentos que foram condicionados com o extrato foliar da moringa (MLE 1:30). Constatou-se que a temperatura de 10/5 °C por oito dias causa estresse irreversível para o aparato fotossintético e membranas celulares de moringa, após quatro dias de recuperação em condições ótimas. A temperatura letal de congelamento que causa 50% de injúria nos folíolos de moringa (LT_{50}) foi de -2.8 °C, no entanto a espécie não apresentou habilidade de aclimatação. No estudo sobre a diversidade genética dos acessos do BAG de Moringa, os 20 primers ISSR amplificaram 144 bandas, na qual 100% delas foram polimórficas. Os valores médios da heterozigose esperada (He) e do Índice de Shannon (I) foram de 0.11 e 0.12, respectivamente. Os genótipos foram divididos em dois grupos de acordo com a análise do programa STRUCTURE. Por fim, os resultados demonstraram que o BAG de Moringa apresentou baixa diversidade genética.

Palavras-chave: germinação de sementes, extrato foliar de moringa, estresse de baixas temperaturas, ISSR.

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* Comitê Orientador: Dra. Ana Veruska Cruz da Silva – UFS (Orientadora), Dr. Evandro Neves Muniz – Embrapa (Coorientador).

ABSTRACT

SOARES, Tássia Fernanda Santos Neri. **Seed treatment, stress tolerance, and genetic variability of *Moringa oleifera* Lam.** São Cristóvão: UFS, 2023. 82p. (Thesis - Doctor of Science in Agriculture and Biodiversity).*

The tress species *Moringa oleifera* Lam. is popularly known for its multiple-purpose applications. In view of the high nutritional value of the leaves, moringa can be used for animal as well human food. The species is native to India and widespread in tropical and subtropical regions around the world. The purpose of this study was to: (1) discuss the importance of the moringa leaf extract and assess its efficiency in moringa seed treatment; (2) evaluate the tolerance of the species to low temperatures stress; and (3) estimate the genetic diversity of the Active Moringa Germplasm Bank of Embrapa Tabuleiros Costeiros. To this end, the moringa leaf extract (MLE 1:30) and the other treatments (soaking in wate; 24-epibrassinolide (EBL 10^{-10}, 10^{-8}, and 10^{-6} M) and ascorbic acid (AsA 50, 100, and 150 mg.L-1)) were applied to moringa seeds and germination and growth were evaluated. To determine cold tolerance, moringa seedlings were cooled to temperatures of 20/15, 15/10 and 10/5 ℃ (day/night) for 7 and 14 days. Thereafter, the effects of stress duration and the recovery under optimal conditions were evaluated by means of morphological and physiological parameters of the seedlings. In addition, the ability of the species to tolerate negative temperatures was evaluated based on a freezing - thawing protocol and the capacity to acclimatize was tested. The genetic diversity in 25 accessions derived from 177 Moringa BAG genotypes was estimated using 20 ISSR markers. It was observed that the seed treatments favored moringa germination and seedling growth. Furthermore, the results of morphological and physiological parameters improved when seeds were treated with MLE 1:30. After eight days at 10/5 ℃ and four days of recovery under optimal conditions, the damages to the photosynthetic apparatus and cell membranes of moringa were found to be irreversible. The lethal freezing temperature that causes 50% injury to the moringa leaflets (LT50) was -2.8 ℃, and no ability of the species to acclimatize could be observed. In the study on the genetic diversity of the Moringa BAG accessions, the 20 ISSR primers amplified 144 bands, of which 100% were polymorphic. The mean values of the expected heterozygous (He) and Shannon Index (I) were 0.11 and 0.12, respectively. STRUCTURE program analysis divided the genotypes into two groups. Finally, the results allowed the conclusion that the genetic diversity in the Moringa BAG is low.

Keywords: seed germination; moringa leaf extract; low temperature stress; ISSR.

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* Advisory Committee: Dr. Ana Veruska Cruz da Silva - UFS (Advisor), Dr. Evandro Neves Muniz - Embrapa (Co-advisor).

1. INTRODUÇÃO GERAL

A *Moringa oleifera* Lam. popularmente conhecida como moringa, é uma espécie perene de folha caduca, nativa da Índia e amplamente cultivada em regiões tropicais e subtropicais ao redor do mundo (PANDEY *et al.*, 2011). A moringa se destaca pelos seus diversos usos, na qual suas sementes que devido ao alto conteúdo de óleo pode ser usada como uma possível fonte de biocombustível, na alimentação humana, na indústria de cosméticos e na purificação de água (LEONE *et al.*, 2015).

Além disso, diante de um cenário de mudanças climáticas, a moringa vem ganhando destaque como uma espécie potencial para garantir a segurança alimentar e enfrentar a destruição em países subdesenvolvidos (BOOPATHI, RAVEENDRAN E KOLE, 2021). Diversas partes da moringa vem sendo historicamente utilizadas na culinária indiana, podendo suas folhas serem consumidas frescas ou secas na forma de temperos, ou ainda suas vagens como ingredientes de sopas (PANDEY *et al.*, 2011). Além de suas propriedades nutricionais, a moringa se destaca pelas suas propriedades antioxidantes, anti-inflamatórias, antimicrobianas, cardioprotetoras e antidiabéticas (MILLA, PEÑALVER E NIETO, 2021).

Outra utilização dessa espécie que vem ganhando relevância é o uso do seu extrato foliar como um bioestimulante para o crescimento de plantas (ABDEL-RAHMAN E ABDEL-KADER, 2020; JAIN *et al.*, 2020). Tal fato ocorre devido à composição química de suas folhas, compostas por macro e micronutrientes, aminoácidos, antioxidantes tais como flavonoides, ácido ascórbico e hormônios vegetais, como citocinina, auxina e giberelina (LATIF E MOHAMED, 2016). O extrato foliar de moringa se mostrou eficiente para aumentar não só a germinação e o vigor (BIBI *et al.*, 2016), mas também a produção de plantas de diferentes espécies de interesse agronômico, tais como trigo (KHAN *et al.*, 2017), girassol (IQBAL *et al.*, 2020), tomate (BASRA E LOVATT, 2016) e citrus (NASIR *et al.*, 2016). Além disso, esse produto apresenta uma boa alternativa para minimizar o efeito causado por diferentes estresses ambientais por promover aumento da atividade do sistema antioxidante e assim atuar na contenção das espécies reativas de oxigênio (RADY, VARMA E HOWLADAR, 2013). Vale destacar também a versatilidade de utilização desse produto, que pode ser aplicado de forma foliar e no tratamento de sementes (BAKHTAVAR *et al.*, 2015). Assim, o condicionamento de sementes com o uso do extrato foliar de moringa consiste em uma abordagem interessante por se tratar de um produto de baixo custo e sustentável que pode ser utilizado por pequenos agricultores (NOUMAN *et al.*, 2012).

Um dos aspectos limitantes para o crescimento e a produção da moringa é a temperatura, na qual temperaturas acima de 25 °C são consideradas as mais favoráveis (MUHL, TOIT, DU E ROBBERTSE, 2011). Assim, ainda são poucos os estudos na literatura que exploram a tolerância baixas temperaturas para essa espécie. O estresse causado por baixas temperaturas pode ser dividido em estresse de frio, no qual são utilizadas temperaturas de 0 a 20 °C, e o estresse de congelamento com o uso de temperaturas abaixo de 0 °C (Nilsen e Orcutt, 1996). Ambos os estresses, em espécies que são consideradas suscetíveis, levam a danos na estabilidade das membranas celulares e do aparato fotossintético, que dependem da intensidade e duração do estresse (LYONS, 1973). No entanto, essa tolerância ao estresse de baixas temperaturas também pode ser aumentada por meio do processo de aclimatação, a qual consiste na exposição das plantas a temperaturas de frio por um determinado período antes da exposição ao estresse propriamente dito (HINCHA e ZUTHER, 2020).

Diante das diferentes utilizações e potencialidades da moringa, é preciso pensar em estratégias para conservar a sua variabilidade genética. Dessa forma, para garantir a variabilidade genética de uma determinada espécie, os bancos de ativos de germoplasma (BAG) se apresentam como uma eficiente estratégia de conservar o patrimônio genético de forma *ex situ* (BORÉM E MIRANDA, 2013). Com isso, o uso de técnicas que avaliam diferenças

genéticas encontradas diretamente no DNA, como é o caso de marcadores moleculares, apresenta grande vantagem para caracterizar e manejar os recursos genéticos de um BAG (COSTA, SPEHAR E SERENO, 2012). A moringa é uma espécie de polinização cruzada que apresenta alta variabilidade quanto às características morfológicas. No entanto, a baixa variabilidade genética encontrada nos germoplasmas disponíveis para essa espécie apresenta um fator que dificulta o desenvolvimento de seus programas de melhoramento (JATTAN *et al.*, 2021). Nesse sentido, a Embrapa Tabuleiros Costeiros, em 2009, estabeleceu o Banco Ativo de Germoplasma da Moringa, localizado em Nossa Senhora das Dores, Sergipe, Brasil, e que atualmente conta com 25 acessos representados por 177 genótipos, com o intuito de preservar o material genético dessa espécie.

Sendo assim, esse estudo teve como objetivos: (1) discutir a importância do extrato foliar de moringa e verificar a sua eficiência no tratamento de sementes; (2) avaliar a tolerância dessa espécie ao estresse de baixas temperaturas; e (3) estimar a variabilidade genética dos acessos do Banco Ativo de Germoplasma de Moringa da Embrapa Tabuleiros Costeiros.

2. REVISÃO DE LITERATURA

2.1 *Moringa oleifera* Lam.

Moringa oleifera Lam. pertence à ordem Brassicales e à família Moringaceae, a qual contém 13 espécies, sendo esta a mais popular (TRIGO *et al.*, 2021). No Brasil, a *Moringa oleifera* Lam. é também conhecida por moringa, lírio branco, acácia branca ou quiabo-de-quina (BRILHANTE *et al.*, 2017; PEREIRA *et al.*, 2015). Apesar de ser nativa da Índia, a moringa tem sido amplamente cultivada em diversos locais mundialmente, com destaque para o sul da Ásia, África, América Central e América do Sul (PARROTTA, 2009). É bem adaptada a áreas tropicais e subtropicais e se desenvolve favoravelmente em locais com temperaturas médias em torno de 25 a 30 °C (RADOVICH, 2009). Além disso, foi introduzida no Brasil e apresenta boa adaptação às condições climáticas da região Nordeste (GALLÃO, FERNANDES E SOUSA, 2006), sendo considerada uma planta tolerante à seca e que pode ser cultivada em solos arenosos ou argilosos (JATTAN *et al.*, 2021).

A moringa é uma espécie arbórea que possui crescimento rápido e pode atingir cerca de 12 metros de altura, atingindo aproximadamente 1 a 2 metros no primeiro ano, quando cultivada em locais com condições ideais para seu desenvolvimento (PARROTTA, 2009). Seu tronco possui casca cor cinza esbranquiçada e sua copa é aberta com folhas tripinadas composta por folíolos (PANDIYAN *et al.*, 2020). Normalmente, seu florescimento se inicia no primeiro ano após o plantio, no qual produz flores perfumadas com pétalas brancas e estrias amarelas na base (PANDEY *et al.*, 2011). Seus frutos são em forma de vagens longas amarronzadas. Suas sementes são marrons, globulares com cerca de 1 cm de diâmetro com três asas equidistantes que facilitam sua dispersão pelo vento (CAVALCANTE *et al.*, 2017).

Essa espécie é reconhecida pelos seus múltiplos usos, na qual cada parte possui um uso específico. Suas sementes podem ser utilizadas para purificação de água devido as suas propriedades coagulantes que fazem decantar impurezas de forma eficiente e de baixo custo. Isso acontece devido ao pó obtido das sementes ser carregado de partículas positivas que se aderem aos microrganismos e impurezas que normalmente são carregados negativamente (RADOVICH, 2009). Além disso, as sementes de moringa possuem alto teor de óleo, cerca de 42%, também conhecido como óleo "Ben" e pode ser utilizado na alimentação como substituto do azeite de oliva. O óleo obtido das sementes também apresenta forte aplicação na indústria cosmética, sendo ingrediente importante para fabricação de produtos como loções hidratantes e perfumes (FOIDL, MAKKAR E BECKER, 2001).

Diante do seu elevado conteúdo nutricional, as folhas de moringa podem ser utilizadas na alimentação humana e animal (LEONE *et al.*, 2015). Dessa forma, essa espécie vem sendo considerada um superalimento que além de ser rica em nutrientes, é facilmente acessível (BOOPATHI, RAVEENDRAN E KOLE, 2021). As folhas de moringa são caracterizadas por ser uma ótima fonte de proteínas, cerca de 30% do seu peso, alto teor de fibras e baixo teor de gordura. Além disso, estão presentes aminoácidos, vitaminas, minerais e moléculas bioativas, tais como betacaroteno, flavonoides, tocoferol, ácido gálico etc. (MILLA, PEÑALVER E NIETO, 2021).

Na alimentação humana, as folhas de moringa vêm sendo utilizadas como uma hortaliça folhosa, podendo ser ingeridas frescas em saladas ou sucos, cozidas fazendo parte de sopas, secas fazendo parte de chás ou até mesmo em pó que pode ser utilizado em diferentes receitas. Em países africanos, o uso de suas folhas tem sido incentivado como um forte aliado na dieta de mulheres grávidas e no tratamento de crianças com desnutrição (PRICE, 2007). Além das folhas, as vagens de moringa também são utilizadas popularmente como um vegetal na culinária indiana (PANDIYAN *et al.*, 2020). É importante ressaltar que apesar de todas as partes da planta serem consideradas comestíveis, suas raízes e casca não são seguros para ingestão devido a sua composição (MILLA, PEÑALVER E NIETO, 2021).

Na alimentação animal, a moringa pode ser usada para suplementar a alimentação de bovinos, caprinos e aves. Diante da sua composição nutricional, essa espécie se mostrou eficiente não só para aumentar o crescimento, mas também por promover melhorias na qualidade e sabor da carne para consumo humano (GRANELLA *et al.*, 2021). De forma similar, a moringa também tem sido utilizada como um bioestimulante natural para promover o crescimento de plantas e auxiliar a reduzir os efeitos negativos de estresses ambientais (ABD EL-MAGEED, SEMIDA E RADY, 2017).

Por fim, a moringa vem sendo popularmente considerada também como uma espécie medicinal devido aos seus componentes (GOPALAKRISHNAN, DORIYA E KUMAR, 2016). Cerca de mais de 200 compostos foram identificados nessa espécie, dentre eles hidrocarbonetos, cetonas, ácidos graxos, aldeídos, terpenos, dentre outros (FALOWO *et al.*, 2018). Suas folhas se destacam pela variedade de compostos bioativos como vitamina A, B1, B2, B3, C e E, flavonoides, ácidos fenólicos, alcaloides, glucosinalatos e isotiocianatos (LEONE *et al.*, 2015). Sendo assim, diante dos seus compostos químicos, a moringa é considerada promissora para utilização na área farmacológica, principalmente devido a seu potencial anti-inflamatório, antioxidante e antimicrobiano (BRILHANTE *et al.*, 2017).

2.2 Extrato foliar de Moringa e tratamento de sementes

O uso de bioestimulantes naturais tem sido incentivado a fim de encontrar outras soluções para reduzir o uso de fertilizantes inorgânicos. Assim, o uso de produtos naturais pode ser considerado uma alternativa ecologicamente correta e financeiramente acessível para proporcionar incremento no crescimento das plantas (JAIN *et al.*, 2020). Dessa forma, o extrato foliar de moringa surge como um dos bioestimulantes naturais mais promissores por serem usados de forma sustentável para aumentar a qualidade e a produção das plantas (ZULFIQAR *et al.*, 2020). Tal fato é reconhecido devido à grande variedade de macro e micronutrientes presentes nas folhas de moringa, tais como N, Ca, K, Mg, Fe, Ni, Mn, Zn, Co, Bo e S (ABD EL-MAGEED, SEMIDA E RADY, 2017; ALI, HASSAN E ELGIMABI, 2018; NOUMAN, MAQSOOD E BASRA, 2014; REHMAN *et al.*, 2017). Além disso, o extrato foliar de moringa é caracterizado pela elevada quantidade de antioxidantes como ácido ascórbico, glutationa e tocoferol (ABD EL-MAGEED, SEMIDA E RADY, 2017), e pela elevada presença de hormônios de crescimento vegetal, principalmente citocinina, giberelina e auxina (RADY, VARMA E HOWLADAR, 2013).

Na literatura, o uso do extrato foliar de moringa tem sido reportado em trabalhos para superação dos efeitos negativos causado por condições estressantes, tais como altas temperaturas (AFZAL *et al.*, 2020), estresse salino (YASMEEN *et al.*, 2013), estresse hídrico (PERVEZ *et al.*, 2017) e estresse causado por metais pesados, como o mercúrio (BIBI *et al.*, 2016) e cádmio (KHALOFAH *et al.*, 2020). No entanto, o extrato foliar de moringa também tem sido utilizado em condições ambientais ótimas para aumento da germinação e melhor estabelecimento de plantas para promover aumento na performance e produtividade (BASRA, IFTIKHAR E AFZAL, 2011; IQBAL, 2015; KHAN *et al.*, 2017). O extrato foliar de moringa pode ser aplicado de diferentes formas, via foliar (ABDEL-RAHMAN E ABDEL-KADER, 2020), em brotos (AHMAD *et al.*, 2019) e no tratamento de sementes (BAKHTAVAR *et al.*, 2015).

O condicionamento de sementes é uma forma de tratamento de sementes que consiste na hidratação controlada até que seja atingido o metabolismo pré-germinativo, mas evitando a protrusão radicular. Ao final desse processo, as sementes são submetidas à secagem natural até retornar ao ponto de umidade ideal a fim de serem armazenadas (FAROOQ *et al.*, 2009). Durante o condicionamento ocorre a embebição das sementes que ativa rotas metabólicas para o início dos mecanismos de reparo de DNA e nova síntese de antioxidantes. Consequentemente, quando as sementes são colocadas para secagem até atingirem o teor de umidade anterior à

embebição, o metabolismo pré-germinativo é preservado e pode ser recrutado após a nova hidratação quando acontece a germinação propriamente dita (PAPARELLA *et al.*, 2015). Tal fato reflete no aumento das taxas de geminação e emergência, além de conferir melhorias no vigor e uniformidade (FAROOQ *et al.*, 2019; REHMAN *et al.*, 2015; SHARMA *et al.*, 2014). Além disso, a possibilidade de utilização dessa técnica de forma simples e acessível aos produtores consiste em uma relevante vantagem do condicionamento de sementes como uma forma sustentável de aumentar velocidade de germinação e de proporcionar melhor estabelecimento em campo (CARRILLO-RECHE, VALLEJO-MARÍN E QUILLIAM, 2018).

Existem diferentes abordagens para o uso da técnica de condicionamento de sementes, dentre elas está o hidrocondicionamento (embebição das sementes usando somente água) e o condicionamento hormonal (embebição das sementes em soluções com reguladores de crescimento) (JISHA, VIJAYAKUMARI E PUTHUR, 2013). Além disso, o extrato de determinadas plantas, como o extrato foliar de moringa, tem sido utilizado com sucesso no condicionamento de sementes ao invés da aplicação de substâncias comerciais como reguladores de crescimento para atingir o mesmo objetivo das abordagens anteriormente mencionadas (FAROOQ *et al.*, 2019).

Dentre os reguladores de crescimento que vem sendo utilizados no condicionamento de sementes, destaca-se o 24-epibrassionolideo. Esse regulador de crescimento é classificado como um tipo de brassinosteroides que é reconhecido por atuar em diferentes funções do crescimento das plantas, desde a germinação de sementes até o crescimento do tubo polínico, o alongamento do caule e conferir resistência a estresses (NAWAZ *et al.*, 2017; VARDHINI E ANJUM, 2015). O 24-epibrassionolídeo tem sido amplamente utilizado no tratamento de sementes de diferentes espécies olerícolas como a mostarda (CHOUDHARY *et al.*, 2011; SOARES *et al.*, 2020), o tomate (AHAMMED *et al.*, 2012) e o pimentão (SILVA *et al.*, 2015). No entanto, ainda são poucos os estudos que abordam o uso da aplicação exógena desse hormônio vegetal em espécies florestais (KUNEŠ *et al.*, 2017).

De forma similar, o ácido ascórbico também vem sendo utilizado no condicionamento de sementes. Popularmente conhecido como Vitamina C, o ácido ascórbico é considerado um dos antioxidantes mais abundantes encontrados nas plantas (RAFIQUE *et al.*, 2011; REIAHI E FARAHBAKKSH, 2013). O ácido ascórbico atua como composto não enzimático na defesa contra a produção de radicais livres que induzem o processo oxidativo (HUSSAIN *et al.*, 2017). Assim, a aplicação do ácido ascórbico é reconhecida também por promover aumento da viabilidade e do vigor de sementes (BRILHANTE *et al.*, 2013).

A moringa pode ser propagada tanto vegetativamente como por sementes. Pode ser adotado o método de semeadura direta bem como a produção de mudas em sacos plásticos. Para fins comerciais em larga escala, a moringa pode ser plantada em cultivo intensivo, visando a produção de folhas. Sua germinação ocorre entre 5 e 12 dias após a semeadura (GOPALAKRISHNAN, DORIYA E KUMAR, 2016). O uso de sementes de moringa de alta qualidade é de fundamental importância para o estabelecimento de um estande uniforme e o uso da técnica de condicionamento pode ser eficiente aliada para realçar a qualidade das sementes nesse sentido (MARCOS-FILHO, 2015). O uso da técnica de condicionamento vem sendo utilizada para essa espécie não só para induzir resistência a estresses ambientais (NOUMAN, MAQSOOD E BASRA, 2014), mas também para aumentar e uniformizar a germinação e aumentar o vigor de plântulas sob condições ótimas (NOUMAN *et al.*, 2012).

2.3 Tolerância a estresses de frio e congelamento

Baixas temperaturas são consideradas um dos fatores mais relevantes para a distribuição geográfica das espécies (ALLEN E ORT, 2001). Sendo assim, as plantas podem ser classificadas quanto a sua capacidade de tolerância ao frio. São consideradas sensíveis ao frio

aquelas que sofrem lesões ou não sobrevivem em temperaturas entre 0 e 15-20 ℃, como é o caso das plantas tropicais (NILSEN E ORCUTT, 1996). Já as plantas insensíveis ao frio conseguem se desenvolver e terminar seu ciclo de vida sob baixas temperaturas (RAISON E LYONS, 1986).

De forma geral, a severidade do estresse causado por baixas temperaturas aumenta com a redução da temperatura e com o aumento da duração de exposição a uma determinada temperatura (LYONS, 1973). Além disso, os sintomas do estresse causado pelo frio podem ser manifestados durante o período que a planta está submetida ao resfriamento ou ainda após o reaquecimento do material vegetal. Sendo assim, faz-se necessário avaliar os efeitos deletérios não só durante o estresse propriamente dito, mas também quando as plantas retornam para condições ambientais ótimas (NILSEN E ORCUTT, 1996).

O estresse causado por baixas temperaturas pode causar disfunções em estruturas fisiológicas essenciais, como os cloroplastos, no qual não só as membranas, mas também os fotorreceptores são capazes de perceber esses danos (LIU *et al.*, 2018). Assim, dentre um dos efeitos negativos causados pelo estresse de frio está o comprometimento da cadeia de transporte de elétrons durante o processo de fotossíntese. Nesse sentido, a avaliação da fluorescência da clorofila por meio do parâmetro Fv/Fm é relevante para identificar danos causados no fotossistema II, que podem ser ou não irreversíveis (ALLEN E ORT, 2001). O índice de Fv/Fm representa a eficiência fotossintética máxima, obtido de forma não destrutiva, no qual é considerado eficiente para avaliar a tolerância a baixas temperaturas (ZAREEI *et al.*, 2021).

O estresse de congelamento é um processo físico no qual as plantas são submetidas a temperaturas inferiores a zero (NILSEN E ORCUTT, 1996). Durante esse processo, podem ocorrer lesões nos tecidos devido à formação de cristais de gelo que culminam na desidratação e podem ser ou não irreversíveis (PETRUCCELLI *et al.*, 2022). Isso ocorre devido ao gelo extracelular que decai o potencial hídrico fora da célula, o que leva a movimentação de água do citoplasma para a membrana (XIN E BROWSE, 2000). O processo de resfriamento lento é desejável para possibilitar a difusão da água extracelular de forma a ter um equilíbrio entre o protoplasma e assim permitir que a formação do gelo extracelular não comprometa permanentemente as funções de membrana (ARORA, 2018).

Uma das formas de avaliar a capacidade da planta de tolerar o congelamento é por meio da definição do LT_{50}, que consiste na temperatura letal capaz de provocar 50% de morte da planta (LIM, ARORA E TOWNSEND, 1998). Para isso, foram determinados protocolos artificiais de congelamento e descongelamento, no qual um tecido vegetal é submetido ao resfriamento por determinadas temperaturas abaixo de zero a uma taxa fixa de 1h/℃, seguido por uma nucleação de gelo com posterior descongelamento a 0-4 ℃ durante aproximadamente 12-20h (MIN, CHEN E ARORA, 2014). Após isso, avalia-se a injúria causada na integridade das membranas por meio da quantificação do extravasamento de íons (LIM, ARORA E TOWNSEND, 1998). Esse protocolo tem sido amplamente utilizado em diferentes espécies como cebola (CHEN *et al.*, 2013), espinafre (MIN, CHEN E ARORA, 2014), pêssego (ARORA E WISNIEWSKI, 1992) e goiaba (HAO *et al.*, 2009), com o objetivo de simular um evento de geada que ocorre em condições naturais (MIN, CHEN E ARORA, 2014). Assim, seja em condições naturais ou em laboratório, não só o congelamento contribui para ocorrência de lesões nos tecidos das plantas, mas também o descongelamento (CHEN, FESSEHAIE E ARORA, 2012).

A tolerância ao congelamento pode ser aumentada por meio do processo de aclimatação, no qual as plantas são expostas por um determinado período a baixas temperaturas, porém acima do ponto de congelamento (HAO *et al.*, 2009). Nesse sentido, a aclimatação consiste em um processo complexo no qual reajustes fisiológicos e moleculares estão envolvidos para permitir o posterior aumento da tolerância ao congelamento (PETRUCCELLI *et al.*, 2022). Um dos primeiros locais que ocorrem danos causados pelo congelamento são as membranas

celulares, sendo assim, o processo de aclimatação confere modificações na composição lipídica das membranas. Além disso, promove o acúmulo de solutos e o aumento da atividade antioxidante (XIN E BROWSE, 2000). Esse processo de aclimatação é característico de espécies que são tolerantes ao congelamento, no entanto, essa capacidade também pode ser observada em determinadas espécies que são classificadas como sensíveis ao frio, como é o caso do tomate (BARRERO-GIL *et al.*, 2016).

A moringa é uma espécie característica de climas tropicais e subtropicais, sendo considerada sensível a baixas temperaturas (TRIGO *et al.*, 2021). No Brasil, a moringa é bem adaptada às condições climáticas da região Nordeste. No entanto, reporta-se que seu desenvolvimento inicial pode ser comprometido quando cultivada em regiões do sul do Brasil durante o inverno, no qual é caracterizado pelo clima subtropical com temperaturas médias em torno de 16 ℃ (COSTA *et al.*, 2015). Na literatura reporta-se que a temperatura ideal para o seu crescimento é entre 25 e 35 ℃, sendo que nos meses mais frios pode resistir a poucas e leves geadas entre -1 e -3 ℃ (GODINO, ARIAS E IZQUIERDO, 2017). No entanto, essas informações consistem apenas de relatos e não foi identificado nenhum estudo que avalie sistematicamente se essa espécie pode ou não tolerar a formação de gelo em seus tecidos.

2.4 Variabilidade genética

Com a crescente degradação dos ecossistemas em todo o mundo, faz-se necessário o uso de estratégias para salvaguardar a diversidade genética de populações arbóreas. Nesse contexto, o uso de bancos de germoplasma consiste em uma eficiente forma de conservar o material genético de espécies florestais visando a conservação e manutenção da biodiversidade e base genética (RIBEIRO *et al.*, 2016). Os bancos ativos de germoplasma são um importante instrumento para conservação *ex situ* da variabilidade genética de uma espécie. Assim, com o uso de um determinado número de acessos é possível representar e caracterizar o material genético de uma espécie para conservá-lo a médio e longo prazo, além de promover o intercâmbio desse material entre instituições de pesquisas (COSTA, SPEHAR E SERENO, 2012).

Com o início da tecnologia de marcadores moleculares foi possível avaliar a variabilidade genética dentro e entre as espécies de forma mais rápida e eficiente, e desse modo explorar o potencial genético de cada espécie. Além disso, os marcadores moleculares possibilitam a avaliação de alta quantidade de genótipos com o uso de técnica simples que auxilia a identificar características de interesse para programas de melhoramento genético (BERED, BARBOSA NETO E CARVALHO, 1997). O uso de marcadores moleculares é também de fundamental importância para o manejo dos bancos de germoplasma e possibilitam a detecção de polimorfismo genético diretamente no DNA. Assim, pode-se avaliar o genótipo sem a influência ambiental e obter um número quase ilimitado de polimorfismo genético (COSTA, SPEHAR E SERENO, 2012).

No final da década de 80, as técnicas de PCR (*Polymerase Chain Reaction*) possibilitaram a utilização de marcadores baseados em DNA de forma mais confiável e eficiente, possibilitando uma maior automação das análises (HENRY, 2012). Assim, os marcadores moleculares ISSR (*Inter Simple Sequence Repeat*) são utilizados com base em técnicas de PCR e não requerem informação prévia da sequência do DNA. Eles amplificam segmentos de DNA que ficam entre duas regiões de microssatélites, são reconhecidos por possuírem alta reprodutibilidade e por sua fácil utilização (GODWIN, AITKEN E SMITH, 1997)

Os marcadores ISSR consistem em um dos tipos de marcadores dominantes que codificam fragmentos moleculares e produzem dados binários. A partir disso, é possível obter dados para a estimativa de índices de distância genética entre cada par de acesso e assim criar análises de agrupamento e obter informações mais robustas quanto à caracterização do material

genético (COSTA, SPEHAR E SERENO, 2012). Assim, os métodos de agrupamento por meio de *clusters* fornecem informações que são facilmente visualizadas para identificar indivíduos que são geneticamente aparentados (PRITCHARD, STEPHENS E DONNELLY, 2000).

A moringa é uma árvore de polinização cruzada e sua alta variabilidade parece ser um fator positivo para aperfeiçoar os programas de melhoramento desta espécie (LEONE *et al.*, 2015). No entanto, apesar da moringa ser referida como uma espécie de alta variabilidade, essa variabilidade não é refletida nos bancos de germoplasma, o que dificulta o aprimoramento dos programas de melhoramento da espécie (JATTAN *et al.*, 2021). Além disso, ainda há um pequeno número de variedades lançadas ao redor do mundo, sendo as mais conhecidas a PKM-1 e PKM-2 (RADOVICH, 2009). Na literatura, o uso de marcadores ISSR é reportado como uma forma eficiente para avaliar a variabilidade genética de genótipos de moringa (HASSANEIN E AL-SOQEER, 2018). Tais marcadores ISSR foram ainda considerados mais adequados para avaliar a diversidade genética em *M. oleifera* cultivada na Índia, comparado aos marcadores RFLP e ao RAPD (SAINI *et al.*, 2013).

Em 2009, a Embrapa Tabuleiros Costeiros estabeleceu o Banco de Germoplasma Ativo de Moringa, na qual a primeira caracterização desses 16 acessos foi realizada por meio do uso de marcadores RAPD (SILVA *et al.*, 2012). Desde então, novos acessos foram adicionados ao BAG de Moringa, sendo assim, faz-se necessário uma nova caracterização molecular desse material.

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4. MANUSCRIPT 1
MORINGA LEAF EXTRACT: A COST-EFFECTIVE AND SUSTAINABLE PRODUCT TO IMPROVE PLANT GROWTH

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ABSTRACT
Moringa leaf extract: a cost-effective and sustainable product to improve plant growth

Given the necessity to find new alternatives as a biostimulant to improve plant growth, Moringa Leaf Extract (MLE) has been noticed by the scientific community as being a low-cost and natural product. Several studies have revealed the positive effects of MLE to enhance seed germination, seedling growth, photosynthetic pigments, antioxidant system, yield, and also confer resistance to a variety of abiotic stresses, such as drought, salinity, adverse temperatures, and heavy metals. However, we still require to investigate deeply the MLE composition and the different forms available to apply it. Thus, to integrate the current knowledge, this review insight into new approaches to the types of application, concentration utilized, and gathered different types of methodologies to obtain the MLE. In addition, the positive results of MLE to reduce the effects caused by abiotic stresses were discussed. Future studies should focus on identifying new varieties of Moringa to improve the quality of MLE and explore studies of MLE application not only in crop species but also in forest species.

Key-words: abiotic stresses; foliar spray; seed treatment; plant growth regulators.

4.1. Introduction

Moringa belongs to the Moringaceae family, composed of 13 species, of which *M. pelegrina* and *M. oleifera* (popular known as drumstick or miracle tree) are the most known wide world. Native to India and well spread over Africa, China, Mexico, and the Middle East, Moringa is a fast-growing and perennial tree. Moringa is rising as a plant of multiple uses, being recommended for reforestation programs in India (Farooq e Koul, 2020). Its high content of protein in leaves and seeds, make them a valuable product to human and animal food. Moreover, Moringa seeds have a high content of oil that can be used not only for consumption but also as a potential fuel. Another use of moringa seeds is for water purification, given its flocculant's properties. Moringa seeds after oil extraction process have proteins that are active cationic polyelectrolytes which neutralize the colloids in dirty water. Therefore, the protein of moringa seeds can be used as a non-natural polypeptide to sediment particles due to the negative electrical charge of the colloids (Foidl et al., 2001). Moringa is also considered a medicinal plant because it has several bioactive compounds that may provide anti-inflammatory, antibacterial, and antioxidative properties (Jain et al., 2020). Moringa leaves are composed of range of bioactive compounds, such as vitamin A, B1 (thiamine), B2 (riboflavin), B3 (Niacin), C (ascorbic acid), E (tocopherol), carotenoids (β-carotene and lutein), total phenols, phenolic acids (caffeic acid, galic acid, ferulic acid, o-coumaric acid, ρ-coumaric acid, and chlorogenic acid), and flavonoids (apigenin, kaempferol, leteolin, myricetin, and quercetin) (Leone *et al.*, 2015).

As increasing the world population, the demand for food production increases as well. In agriculture, the necessity to find new products to improve the yield and food quality is also under pressure of the market. With the concern of food security, organic farming has been emerging as an alternative to reduce the use of synthetic growth promoters, fertilizers, and pesticides (Basra and Lovatt, 2016). Hence, natural biostimulants are products obtained by plants that improve different aspects of plant growth, for instance, from seed germination to post-harvest (Zulfiqar *et al.*, 2020). In this context, Moringa Leaf Extract (MLE) is getting the attention of the scientific community as a biostimulant for being equal or more effective than synthetic growth promoters, and mainly due to its low-cost (Khan, Basra, Afzal, Nawaz, *et al.*, 2017b). For the farmers, MLE could be an up-and-coming alternative to avoid using expensive

inorganic fertilizers, and besides that, to preserve the soil and even the farmers' health (Jain *et al.*, 2020).

The use of MLE as a potential growth stimulant has reported better results compared to those using synthetic plant growth promoters such as benzyladenina, a cytokinin type (Abdel-Rahman and Abdel-Kader, 2020), and salicylic acid (Khan, Basra, Afzal and Wahid, 2017b) or even using other plant-based natural growth enhancers such as sorghum water extract (Afzal *et al.*, 2020). The overall effects of MLE on plant growth are related to the high content of minerals, antioxidants, and hormones, mainly cytokinin, auxins, and gibberellins. In contrast to synthetic growth promoters, MLE can provide all those elements at a low-cost and easy to get way (Ahmad *et al.*, 2019). In spite of the MLE benefits being already known, its uses are still not widespread in the industry (Basra and Lovatt, 2016). Moreover, the popularity of MLE as a biostimulant has also been increased due to its uses to deal with environmental stresses conditions that may impair plant growth, such as drought, salinity, high temperatures, and heavy metals (Batool et al., 2020; Latif and Mohamed, 2016). Due to the overview aforementioned, this study aimed to discuss the composition and different forms available to apply MLE, as well as the positive results of MLE to reduce the effects caused by abiotic stresses.

4.2. Constitution of MLE

Moringa leaves are constitute of the following mineral elements: Na, Ca, K, Mg, Fe, Ni, Mn, Zn and Co (Bibi *et al.*, 2016). Plants require them to maintain their plant growth, as well as to improve the synthesis of carbohydrates or even to confer resistance to diseases (Jain *et al.*, 2020). Moreover, MLE contains not only minerals but also antioxidants such as proline, phenols, carotenoids, ascorbic acid, and osmoprotectants, such as amino acids, soluble sugars, and K (Rady, Varma and Howladar, 2013). The MLE also takes into account a great amount of DPPH radical-scavenging activity that reduces the cell membranes damages caused by the peroxidation lipid due to the stress oxidative (Abd El-Mageed et al., 2017). Moringa leaves are also constituted by plant hormones, for instance, auxin, gibberellins, cytokinin, salicylic acid, jasmonic acid, and abscisic acid (Ali et al., 2018). Furthermore, MLE is recognized for acting along with the endogenous plant hormones (Nouman, Siddiqui e Basra, 2012a). Plant growth regulators balance the plant growth by controlling cell division and expansion. They have plenty of uses in horticultural crops, providing benefits for flowering, pre and postharvest to increase the quality of fruits, and reduce damages caused by adverse climatic conditions (Basra and Lovatt, 2016). The elemental composition of MLE is summarized in the Table 1.

The MLE was more promissory to improve vegetative growth and yield of fennel plants when compared to the treatment with benzyladenine (BA), a cytokinin type (Abdel-Rahman and Abdel-Kader, 2020). Basra and Lovatt (2016) reported the presence of different cytokinin nucleosides (iPA, t-ZR, c-ZR and dhZR) in the moringa leaves which act as intermediates in cytokinin biosynthesis. It was noteworthy that the content of those nucleosides varied among the period of the year, being some forms not detectable in specific months. Moreover, the auxin was found mainly in conjugated forms with aspartate and glutamate, and gibberellins in the forms of GA_{19} and GA_1. On the other hand, a plant hormone that has inhibition action, ABA, despite low concentration, was also detected in moringa leaves. The presence of ABA in the MLE besides not reducing the growth in tomato plants probably increased the antioxidant activity and proline contents.

The positive effects of MLE on plant growth seem to be not only due to a class of plant hormone, cytokinin, but also the high content of crude proteins, which are extremely important to the expansion of cellular protoplasm (Iqbal *et al.*, 2020). Khan et al. (2017a) found differences in the composition of MLE among Moringa varieties related to the concentration of mineral nutrients, antioxidants, and plant hormones. They reported that Moringa diversity

allows selecting the greatest varieties that have a rich compound source of MLE. Farooq et al (2019) performed a comparative study of MLEs with five different varieties of Moringa (Conventional, PKM-1, PKM-2, Jaffna, and ODC) that are well cultivated in Southern India. The moringa leaves of Jaffna variety exhibited the highest values of total phenolic and flavonoid content and the highest antioxidant activity estimated by the methods using the DDPH and ABTS chromophores. Similarly, Jain et al. (2020) reported that the foliar spray with MLE of Jaffina variety in *Stevia rebaudiana* showed the highest improvement not only in all plant growth parameters, but also in the sativoside and zeatin content, and the mineral content of K, Na, Ca and Li. The authors strongly recommend the MLE of Jaffna variety to improve crop productivity and raise the content of essential nutrients and bioactive compounds.

The potential benefits of MLEs may decrease with storage time and temperature. The highest contents of ascorbic acid and free phenolics were observed in fresh MLE and MLE stored at low temperature (4 °C in refrigerator) for one month. As a result, the storage of MLE affected the germination time and vigor parameters of wheat seedlings, expressed in terms of fresh and dry weight and seedling length, being the fresh MLE the better one. The benefic results of fresh MLE are also highlighted in chlorophyll a and b contents and even in grain yields of wheat plants. These results may be attributed to the decrease of secondary metabolites over time and different storage conditions of MLE. Secondary metabolites play an important role in the activities of endogenous hormones, being crucial to plant growth (Khan et al. 2017a).

4.3. Obtaining, preservation & application of MLE

In literature are reported different methodologies to obtain the crude extract of Moringa leaves. First, the leaves used can be dried (Rady, Varma and Howladar, 2013) or stored overnight at freezing temperatures (Nouman *et al.*, 2012; Yasmeen, Basra *et al.*, 2013b). Second, the leaves are pressed in a locally fabricated machine (Foidl et al., 2001) or homogenized using a household blender. Next, the extract obtained is sieved using a mutton cloth (Abdel-Rahman and Abdel-Kader, 2020), filtered through a paper filter (Whatman No. 1), or centrifugated at 8000 xg for 15 min (Abd El-Mageed et al., 2017; Afzal et al., 2020; Ali et al., 2018). Finally, the extract could be diluted over different concentrations (Nouman et al., 2012b). In addition, the Moringa extract could be diluted in both the aqueous (Ahmad *et al.*, 2019; Ali, Hassan and Elgimabi, 2018; Iqbal *et al.*, 2020; Khan, Basra, Afzal, Nawaz, *et al.*, 2017b) or the ethanolic solution (Abdel-Rahman and Abdel-Kader, 2020; Maishanu et al., 2017). However, in a study comparing them, the aqueous extract showed to be more efficient to improve the growth parameters of fennel plants, in which the concentration at 5 % reached the highest values (Abdel-Rahman and Abdel-Kader, 2020).

Given the rich composition of MLE, it has a variety of forms to apply to plants, which could be on roots (Basra and Lovatt 2016), corms (Ahmad *et al.*, 2019), postharvest preservative solution of cut flowers (Hassan and Fetouh, 2019), or even in postharvest as a potential edible coating (Tesfay and Magwaza, 2017). However, the most popular application of MLE is via foliar spray and in the treatment of seeds. The positive effects of MLE applied on seed treatments showed to increase seed germination, vigor, velocity, synchronicity, and uniformity of germination, besides improving seedling growth that may reflect on many other parameters of plant growth (Khan et al. 2017a). On the other hand, the foliar application of MLE makes plants able to improve their growth by conferring reinforcements in the photosynthetic and antioxidative systems. Other positive effects are related to regulating the water use capacity, and improvements in yield or even increase resistance to abiotic stresses.

The MLE applied to seed priming is attracting the scientific attention to be well regarded in organic agriculture. Despite the variety of products that can be used in seed priming, the high

cost ends up being a problem when it is applied on a large scale. Consequently, the application of MLE on seed priming has been underscored not only for improving seed germination and velocity but also seedling growth and vigor, especially compared to other priming agents (Yasmeen, Basra, Shahzad Maqsood Ahmed, *et al.*, 2013b). In cowpea, the MLE applied in seed priming was the most effective product used to increase the emergence, compared to other techniques such as on-farm priming, hydropriming, and halopriming. The authors suggested that these positives effects may be due to the presence of zeatin and nutrients (Iqbal, 2015). In Moringa plants, the positive effects of MLE applied in seed priming varied among the duration of seed soaking. The maximum values of root length, chlorophyll a, β-Carotene, and mineral contents were reached in plants that had seed priming with MLE for 8h, compared to 16 and 24 h of seed soaking (Nouman *et al.*, 2012).

The treatment with the application of MLE on seed priming along with foliar spray provided the best results in order to improve growth performance and yield of maize plants under early and optimum sowing conditions (Bakhtavar *et al.*, 2015). In addition, the foliar application of MLE along with $ZnSO_4$ and K_2SO_4 increased the quality and yield of mandarin fruits, being considered a great alternative product instead of using synthetic plant growth promoters. Biochemical parameters of mandarin fruits ie., soluble solid contents, titratable acidity, vitamin C, sugars, total phenolics, and total antioxidants showed a significant increase in all treatments with MLE foliar spray (Nasir *et al.*, 2016). Similar results were found in fennel plants, in which the MLE sprayed lead to the production of high fruits and oil yield. Those effects are being mainly attributed to the high content of zeatin in MLE, which plays an important role in the assimilation and translocation of photoassimilates towards fruit (Abdel-Rahman and Abdel-Kader, 2020).

The uses of MLE could be considered as an auspicious product to the flower crop market. MLE showed positive effects on increasing the longevity of cut gladiolus spikes and the number of opened florets. Moreover, the bacterial number was reduced in a vase solution with MLE at a concentration of 3 and 4 %. In addition, it was observed that the production of ROS (Reactive oxygen species) and the MDA (Malondialdehyde) content increased during the floret's life; however, this increase was minimized in treatments with MLE. This study showed an interesting result in order to ameliorate the negative effects of senescence of cut gladiolus spikes by decreasing the oxidative metabolism, antimicrobial activity and preserve the chlorophyll content (Hassan and Fetouh, 2019). The MLE has been also testing in the floriculture crop production, mainly to uniform the corm sprouting and increase the quality of cut stems. Treated corms of *Freesia hydrica* with MLE solution at a concentration of 5 % showed a faster sprouted time as well as an increase in stem growth and chlorophyll contents. Moreover, the application of MLE solutions at a concentration of 3% after 30 or 60 days of planting also increased all plant growth parameters and the number of marketable stems per plant. Another interesting result is that the corms of *Freesia hydrica* soaked in MLE solution showed the greatest yield than the foliar application (Ahmad *et al.*, 2019).

The positive effects of MLE in plant growth can be significantly affected by its concentration and requires to be adjusted by each species, duration, and frequency of treatment and form of application. In *Pelargonium graveolens* L. Her., the growth attributes were evaluated using different concentrations of MLE (1:40, 1:30, 1:20, 1:10), which were diluted with distilled water (v:v) and applied via foliar. This study reported that the plant growth parameters and biomass yield increased as the concentration of MLE increased, reaching the highest values in treatments with MLE 1:20. In addition, at the most concentration rates, it reached the highest the content and composition of volatile oil. Improvements in those traits represent interesting progress to the aromatic plants market to find alternative products as plant growth promoters (Ali, Hassan e Elgimabi, 2018). The foliar spray of MLE raised significantly

yield in the sunflower crop. At the most concentrate solution (50%), MLE provided an improvement to bust the potential growth, compared to the extract obtained by Moringa roots (Iqbal *et al.*, 2020). In contrast, MLE at the concentration diluted in water 30 times was efficient to improve the velocity and synchronized the seed germination of different range grasses (Nouman, Siddiqui e Basra, 2012a). In pea plants, the MLE applied via foliar spray of MLE over different concentrations (1-4 %) increased the plant height and pod lengths (Merwad, 2018). Similarly, the MLE improved the growth and yield in cowpea plants, and the results were better when the frequency of MLE application increased (Maishanu *et al.*, 2017).

The conservation of MLE seems to be a key process, but it must be so to preserve especially the most sensitive agents such as plant hormones and proteins, in addition to other secondary metabolites (hormones are not secondary metabolites). It was reported that the MLE could be stored at room temperature (Iqbal, 2015) or low temperatures (Bibi *et al.*, 2016; Farooq, Hussain, *et al.*, 2019; Rady, Varma C. e Howladar, 2013). The storage of MLE was evaluated in two temperatures regimes (ambient temperature of 30-35 ºC and 4 ºC in a refrigerator), with or without preservative for one, two, and three months (Khan, Basra, Afzal, Nawaz, *et al.*, 2017). The authors concluded that MLE can be stored till 1 month without preservative at ambient temperature and increasing this time implies decreasing the number of bioactive compounds.

4.4. MLE to increase plant tolerance to abiotic stress

Plants could be submitted to a variety of abiotic stress, as an illustration, drought, salinity, chilling or high temperatures, and heavy metals toxicity. As a result, there is an emerging necessity to find non-conventional products, as an example MLE, to alleviate the effects of these stressful conditions in plant growth (Zulfiqar *et al.*, 2020). Abiotic stress can lead plants to reduce drastically its productivity by altering metabolic pathways, mostly related to plant hormone (Wani *et al.*, 2016). Adverse conditions reduce plant growth due to the increase of ROS production that causes damages to the structure of cell membranes and, consequently increases the lipid peroxidation. In addition, the protein synthesis and the antioxidant system could be impaired by those changes (Latif and Mohamed, 2016). The process of overcoming abiotic stress can be associated with the activity of osmoprotectants. To give an instance, proline and soluble sugars act on plant cells to provide the osmotic adjustment by increasing the membrane stability. MLE has demonstrated its efficient role in releasing the negative effects of abiotic stresses (Table 2), mainly due to the presence of minerals, antioxidants, amino acids, and soluble sugars. The application of MLE is recognized for increasing the membrane stability index, providing a healthier metabolic condition that resulted in vigorous plant growth (Batool, Khan e Basra, 2020). When MLE was applied to plants under abiotic stress, the photosynthetic system was reinforced with more production of leaf pigments that raise chlorophyll fluorescence. This fact could provide an increase in osmoprotectants production (Abd El-mageed et al., 2017).

Currently, the use of MLE as a seed treatment product has been rising, especially to overcome abiotic stresses. In the market, there is a low variety of plant growth regulators that are especially recommended to mitigate the effects of abiotic stress conditions on plants, as an illustration, X-Cite® (cytokinins), Activol® (gibberellic acid), and ethrel® (ethylene) (Basra and Lovatt, 2016). Soaking seeds with MLE showed positive results to alleviate the adverse effects caused by NaCl stress on growth and yield of bean plants. The content of MDA that assess the level of lipid peroxidation, and the content of Reactive Oxygen Species (ROS), taken O_2^- and H_2O_2 as an example, showed ameliorate in the treatments with MLE. Ascorbic acid and plant hormones such as auxin, gibberellins, and cytokinins probably supported seedlings to deal with the salinity stress (Rady, Varma C. e Howladar, 2013). Another promising result is observed

using MLE to reduce heavy metal stresses. In maize, seeds pre-treated with MLE mitigated the negative effects of HCl_2 on seed germination and protected root growth. In addition, the chlorophyll and carotenoid content were also increased by soaking maize seeds with MLE. Although the presence of Hg can cause toxicity and lead to growth inhibition, its accumulation was higher in treatments with MLE. The authors suggested that the phenolics detected in MLE can increase the endogenous level of total soluble phenolics, which boosts up the resistance to Hg stress (Bibi *et al.*, 2016b).

The foliar application of MLE has been showing positive results to alleviate the effects on plants under a variety of abiotic conditions, such as low-temperature regimes (Batool, Khan e Basra, 2020), drought (Abd El-Mageed et al., 2017; Pervez et al., 2017), salinity (Yasmeen, Basra, Shahzad Maqsood Ahmed, *et al.*, 2013b), and heavy metal (Howladar, 2014; Khalofah *et al.*, 2020). The negative effects of heat stress were mitigated by using a foliar application of MLE in the wheat crop on a field study. High temperatures caused a reduction in plant growth parameters; however, those effects were overcome by MLE, probably due to the high contents of antioxidants in Moringa leaves that raised the levels of enzymes activity which acts on the antioxidant system such as CAT, APX, and POX (Afzal *et al.*, 2020).

In bean plants, the foliar application of MLE ameliorated the stress effects caused by NaCl, high temperature, and gamma rays on plant growth. In addition, the photosynthetic pigments were repaired and the contents of plant hormones (GA_3, IAA, and CKs) increased in treatments with MLE. The release of these negative stresses effects is related to the decrease of MDA and ROS contents in plants with MLE application. This could be attributed to the MLE composition which is rich in antioxidants. Moreover, the salinity stress caused changes in the leaf ultrastructure, especially in the mesophyll cells. However, it was observed that the application of MLE minimized the negative effects of salinity on leaf mesophyll cells and increased the number of chloroplasts. These results could be probably attributed to a higher content of cytokinins in MLE, which is of the utmost importance to chloroplast functions (Latif and Mohamed, 2016).

4.5. Future Perspectives

The literature clearly shows the positive effects of MLE to improve different aspects of plant growth. Due to its high content of minerals, antioxidants, proteins, and hormones, the well-known proprieties of MLE have been getting scientific attention for being an organic and low-cost product. However, the use of MLE as a commercial product is still limited. Future research that identifies new varieties which have a higher source of interesting compounds is encouraged to explore the genetic diversity of Moringa species. Furthermore, the definition of MLE concentration according to a specific type of application recommended for each species is necessary to become MLE a product with wide use in the industry. Finally, most studies with MLE are performed with crop species, and little is known about the positive effects of MLE on forest species.

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Figures and Tables

Table 1. Elemental composition of MLE.

Component	Value	References
Osmoprotectants		
Amino acids	106.20 mg g^{-1} DW	Rehman et al. 2017
	387.72 mg g^{-1} DW	Ali et al. 2018
	130.00 mg g^{-1} DW	El-Mageed et al., 2017
Proline	21.00 mg g^{-1} DW	Rehman et al. 2017
	33.65 mg g^{-1} DW	Ali et al. 2018
	30.00 mg g^{-1} DW	El-Mageed et al., 2017
Total sugars	352.28 mg g^{-1} DW	Ali et al. 2018
	346.16 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
Total soluble sugars	248.70 mg g^{-1} DW	Rehman et al. 2017
	170.00 mg g^{-1} DW	El-Mageed et al., 2017
Ash	102.00 mg g^{-1} DW	Rehman et al. 2017
Mineral nutrients		
Calcium	28.00 mg g^{-1} DW	Rehman et al. 2017
	15.92 mg g^{-1} DW	Ali et al. 2018
	8.76 mg g^{-1} DW	El-Mageed et al., 2017
	17.08 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
	27.34 mg g^{-1} DW	Nouman et al., 2014
Nitrogen	12.36 mg g^{-1} DW	Ali et al. 2018
	31.30 mg g^{-1} DW	El-Mageed et al., 2017

	13.23 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
Magnesium	6.70 mg g^{-1} DW	Rehman et al. 2017
	3.96 mg g^{-1} DW	Ali et al. 2018
	6.04 mg g^{-1} DW	El-Mageed et al., 2017
	2.98 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
	28.76 mg g^{-1} DW	Nouman et al., 2014
Potassium	25.10 mg g^{-1} DW	Rehman et al. 2017
	13.78 mg g^{-1} DW	Ali et al. 2018
	27.70 mg g^{-1} DW	El-Mageed et al., 2017
	12.45 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
	2.87 mg g^{-1} DW	Nouman et al., 2014
Phosphorus	8.10 mg g^{-1} DW	Rehman et al., 2017
	6.12 mg g^{-1} DW	El-Mageed et al., 2017
	3.82 mg g^{-1} DW	Ali et al., 2018
	3.18 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
	3.23 mg g^{-1} DW	Nouman et al., 2014
Sodium	0.75 mg g^{-1} DW	Rehman et al., 2017
	0.27 mg g^{-1} DW	Nouman et al., 2014
Iron	1.60 mg g^{-1} DW	Rehman et al., 2017
	0.38 mg g^{-1} DW	Ali et al., 2018
	1.89 mg g^{-1} DW	El-Mageed et al., 2017
	0.41 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
	0.44 mg g^{-1} DW	Nouman et al., 2014
Manganese	0.84 mg g^{-1} DW	Rehman et al., 2017
	0.97 mg g^{-1} DW	El-Mageed et al., 2017
	0.03 mg g^{-1} DW	Nouman et al., 2014
Boron	0.02 mg g^{-1} DW	Nouman et al., 2014
Zinc	0.27 mg g^{-1} DW	Rehman et al., 2017
	0.05 mg g^{-1} DW	Ali et al., 2018
	0.45 mg g^{-1} DW	El-Mageed et al., 2017
	0.06 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
	0.03 mg g^{-1} DW	Nouman et al., 2014
Copper	0.14 mg g^{-1} DW	Rehman et al. 2017
	0.03 mg g^{-1} DW	Ali et al., 2018
	0.21 mg g^{-1} DW	El-Mageed et al., 2017
	0.001 mg g^{-1} DW	Nouman et al., 2014
Sulphur	2.68 mg g^{-1} DW	El-Mageed et al., 2017
Bioactive compounds		
Total phenols	1.63 mg g^{-1} DW	Ali et al. 2018
	1.70 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
Soluble phenols	6.20 mg g^{-1} DW	Rehman et al. 2017
Total chlorophyll	4.38 mg g^{-1} DW	Ali et al. 2018

	3.86 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
Total carotenoids	3.10 mg $100g^{-1}$ FW	Rehman et al. 2017
	1.72 mg g^{-1} DW	Ali et al. 2018
	1.65 mg g^{-1} DW	El-Serafy and El-Sheshtawy, 2020
Ascorbic acid	8.47 mg g^{-1} FW	Ali et al. 2018
	2.25 μmol AsA g^{-1} DW	El-Mageed et al., 2017
α-Tocopherol	26.5 μg g^{-1} DW	El-Mageed et al., 2017
Glutathione	0.46 μmol GSH g^{-1} DW	El-Mageed et al., 2017
DPPH radical-scavenging activity	78.4 %	El-Mageed et al., 2017
Super oxide dismutase	190.74 IU min^{-1} mg^{-1} protein	Iqbal et al. 2020
	214.36 unit mg^{-1} protein	Nouman et al., 2014
Peroxidase	20.43 IU min^{-1} mg^{-1} protein	Iqbal et al., 2020
Catalase	72.77 unit mg^{-1} protein	Nouman et al., 2014
Plant hormones		
Indole-3-acetic acid	0.83 μg g^{-1} DW	Rehman et al., 2017
	0.72 μg g^{-1} FW	Ali et al., 2018
Gibberellins	0.74 μg g^{-1} DW	Rehman et al. 2017
	0.65 μg g^{-1} FW	Ali et al. 2018
	2.80 μg g^{-1} DW	El-Mageed et al., 2017
Cytokinin	0.63 μg g^{-1} FW	Ali et al. 2018
	2.24 μg g^{-1} DW	El-Mageed et al., 2017
Zeatin	0.96 μg g^{-1} DW	Rehman et al. 2017
Salicylic acid	1.87 μg g^{-1} FW	Ali et al. 2018
	82.4 μg g^{-1} DW	El-Mageed et al., 2017
Trans-Jasmonic acid	0.20 μg g^{-1} FW	Ali et al. 2018
Abscisic acid	0.29 μg g^{-1} DW	Rehman et al. 2017
	0.13 μg g^{-1} FW	Ali et al. 2018

Table 2. Summary of recent reports on the role of MLE in alleviation of various abiotic stresses in plant.

Plant Species	Type of stress	Form of application	Concentration (w/v)	Responses	References
Zea Mays L.	Temperature	Seed treatment for 24 hours and Foliar spray at knee height, tasseling, and gain filling	Water extract diluted at concentration of 3 %	Improved stand establishment, growth and grain yield	Bakhtavar et al. 2015

Moringa oleifera L.		Foliar spray applied twice at one- and three-month seedling age	Water extract at concentration of 3 %	Increased number of branches, leaves, leaf chlorophyll contents, membrane stability index and leaf phenolic contents	Batool et al. 2019
Zea Mays L.	Mercuric chloride	Seed treatment for 3 hours	Water extract at concentration of 2.5 and 5 %	Alleviated the effects of metal stress on seed germination, seedling growth, chlorophyll, and phenolics content. Enhanced the Hg phytoremediation potential of maize	Bibi et al. 2016
Cucurbita pepo L.	Drought	Foliar spray twice at 20 and 35 days after planting	Water extract at concentration of 3 %	Increased growth and yield characteristics, leaf anatomy, soluble sugars, leaf anatomy, RWC, MSI and WUE	El-Mageed et al. 2017
Phaseolus vulgaris	Salt, high temperatures, and gamma rays	Foliar spray twice at 30 and 37 days after sowing (vegetative stage)	Water extract 30 times diluted	Improved the antioxidative system that reflected on increases of growth, photosynthetic pigments and reduction in MDA and ROS contents	Latif et al. 2016
Phaseolus vulgaris	Salt	Seed treatment for 8 hours	Ethanolic extract and dissolution in 2 l distilled water	Improved growth, yield and antioxidant system	Rady et al. 2013

Phaseolus vulgaris	Salt and Cadmium	Foliar spray twice at 3 and 4 weeks after sowing	Water extract 30 times diluted	Mitigated the stress effects by increasing antioxidant enzymes and proline content. Increased growth, photosynthetic pigments, yield and pod protein	Howladar et al. 2014
Lepidium sativum	Cadmium	Foliar spray three times a week for 21 days starting at 10^{th} DAS	Water extract at concentration of 2, 4, 6, 8 and 10 %	Reduction in MDA and H_2O_2 content and increase in antioxidant enzymes (SOD, CAT, PPO, ASO and GR) and non-enzymatic antioxidant content	Khalofah et al. 2019
Triticum aestivum L.	Salt	Foliar spray at tillering, jointing, booting and heading wheat stage	Water extract 30 times diluted	Activated the antioxidant system and decreased the accumulation of Na^+ and Cl^-	Yasmeen et al. 2013
Zea mays L.	Drought	Foliar spray twice, one at 21days seedling age and the other 6 days after 10 days of drought stress	Water extract diluted at concentration of 12.5, 6.25, 3.12 and 1.56 %.	Improved leaf soluble proteins, leaf relative water content, and plant growth. Reduction of cell wall bound phenolics	Pervez et al. 2016

5. MANUSCRIPT 2
SEED PRIMING AS A STRATEGY TO INCREASE THE PERFORMANCE OF DRUMSTICK TREE

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ABSTRACT
Seed priming as a strategy to increase the performance of drumstick tree

Moringa oleifera Lam. is a multiple purpose tree used as human and animal food, cosmetic production, and water purification. Seed priming combined with growth promoters and natural substances has been used to improve plant performance. This study aimed to verify the efficiency of seed priming using growth-promoting substances such as brassinosteroids, ascorbic acid, and moringa leaf extract on seed germination and seedling growth of *Moringa oleifera*. The seeds were primed with water and 24-epibrassinolide solutions (EBL 10^{-10}, 10^{-8} and 10^{-6} M), ascorbic acid (AsA 50, 100 and 150 $mg.L^{-1}$) and Moringa Leaf Extract (MLE 1:30). Primed seeds with EBL 10^{-8} M improved the speed of seed germination. Seedling length and vigor increased mainly in treatments with AsA 100 $mg.L^{-1}$ and MLE 1:30. The activity of the antioxidant enzyme catalase increased mainly in primed seeds with AsA. Plant height, steam base diameter, number of leaves and gas exchange parameters such as photosynthetic rate, stomatal conductance, and internal CO_2 concentration increased in plants which were from primed seeds with MLE 1:30. Therefore, we recommend seed priming to improve plant growth of *Moringa oleifera*, mainly using MLE, a natural and ecological product.

Key-words: 24-epibrasinolide; ascorbic acid; moringa leaf extract; *Moringa oleifera*.

5.1. Introduction

To improve the performance of any crop under field conditions it is necessary to use high-quality seeds. However, seed quality and viability tend to decline over time due to the deteriorative chemical process that happen in a dry seed (Bewley *et al.*, 2013). This reduction in seed quality can cause a decrease in the speed of germination and provoke uneven germination of a seed lot. As a result, the seed priming technique can be an alternative to increase uniformity and reduce the time for seedling emergence, mainly in low-vigor seeds. Therefore, seed priming consists of controlled hydration to induce the pre-germinative metabolism without root protrusion (Marcos-Filho 2015). During the priming process, the seed is submitted to moderate stress as being hydrated and dried later, which confers a "priming memory", being triggered upon the second hydration. This happens because when a seed is hydrated, events such as mechanisms of Deoxyribonucleic Acid (DNA) repair, mobilization of reserves, and antioxidative system are activated. Hence, this information is preserved as a "priming memory"(Chen and Arora, 2013). The seed priming technique has been used in seed industry, in which patents are released for specialized treatments to improve germination and seedling uniformity emergence (Paparella et al. 2015). On the other hand, seed priming approach is recognized for being a traditional and low-cost technique that farmers around the world have applied to ensure improvements for sustainable plant growth (Carrillo-Reche, Vallejo-Marín and Quilliam, 2018).

Along with priming, growth-promoting substances such as plant hormones can also be used to boost seed germination and plant growth (Jisha et al. 2013). Among them, brassinosteroids (BR) are classified as a new steroidal plant hormone, derived from campesterol and synthesized in young plant tissues. BRs operate in a variety of functions to induce plant

growth, especially in cell division and elongation (Kanwar et al., 2013; Vadhini et al., 2010), and confer resistance to a variety of stress, such as drought (Talaat and Shawky 2016), saline (Dong et al., 2017), high and low temperatures (Jin et al. 2015; Gornik and Lahuta 2017), and heavy metals (Soares et al., 2020). The ascorbic acid is considered another promising product to be used in seed priming (Ahmad et al., 2015). Commonly known as vitamin C, ascorbic acid consists of a non-enzymatic antioxidant that reduces or prevents oxidative stress caused by oxygenated free radicals (Hussain et al., 2017). Ascorbic acid is produced in small amounts and act not only to promote the plant growth by itself but also play a role in biosynthesis of some phytohormones such as gibberellin and ethylene (Kasim et al., 2017).

Similarly, the use of natural products with growth-promoting substances seems to be a relevant and efficient approach to improve plant growth (Bibi et al., 2016; Pervez et al., 2017). For example, Moringa Leaf Extract (MLE) has been used in seed priming as an ecological alternative that farmers can easily adapt (Nouman et al., 2014). MLE is constituted of nutrients, enzymatic and non-enzymatic antioxidants, and plant hormones, such as auxin, cytokinin and gibberellin (Khan et al., 2017; Rady et al., 2013). The effects of MLE on improving germination and plant growth have already been reported in some crops such as maize (Bibi et al., 2016), pea (Iqbal, 2015), wheat (Yasmeen et al., 2013), beans (Howladar, 2014), and grasses (Nouman et al., 2012). Although positive results ensure the efficiency of seed priming application for different agronomic species (Ali, 2017; Li et al., 2017; Nawaz et al., 2016; Yan, 2015), there is a lack of information about seed priming applied to forest species (Missio *et al.*, 2018).

Moringa oleifera Lam., popularly known as drumstick, is the most recognized species among the 13 species of the Moringaceae family (Tshabalala et al., 2019). Also known as the "miracle tree", *Moringa oleifera* is native to northern India and cultivated in a wide range of tropical countries (Leone et al., 2015). Historically, this species is considered a multipurpose tree, with different parts that can be used not only in animal and human nutrition but also in traditional medicine (Jaja-Chimedza et al., 2017). In addition, *Moringa oleifera* seeds have high oil content, called Ben oil that can be used in the food industry, being an alternative to olive oil (Foidl et al., 2001). It can be propagated by seeds or cuttings, being preferable seeds due to the availability and lower costs and labor. Therefore, using seeds with a higher germination rate is an important step for planting success (Leone et al., 2015). Seed priming is recognized for improving germination speed due to the action on DNA repair, ROS signalling, antioxidants activation, early reserve mobilization, endosperm weakening, and altering hormonal regulation, culminating in a greater germination process (Chen and Arora, 2013). In the literature, the use of seed priming in *Moringa oleifera* has been already reported by Nouman et al. (2014), however it only focused on improving plant growth under salinity stress. Thus, this study aimed to verify the efficiency of seed priming using plant growth promoting substances such as brassinosteroids, ascorbic acid, and Moringa Leaf Extract on seed germination, seedling and plant growth of *Moringa oleifera* Lam.

5.2. Material and Methods

5.2.1. Seed priming application

The seeds of *Moringa oleifera* were collected from local matrices trees, located in city of Aracaju, Brazil (-10.944410, -37.075507). Firstly, the seeds were pre-soaked for 12 h (Nouman et al., 2014) in water (hydroconditioning), 24-epibrassinolide (EBL) solutions at concentrations of 10^{-10}, 10^{-8} and 10^{-6} M; ascorbic acid (AsA) at concentrations of 50, 100 and 150 $mg.L^{-1}$; and Moringa Leaf Extract (MLE 1:30). After pre-soaking, the seeds were dried at room temperature until they reached constant weight for 72 hours. The control treatment was composed of seeds without priming.

5.2.2. Preparation and characterization of MLE

The MLE was prepared using young leaves which were overnight frozen and then grinded with a blender to extract its juice. The extract was filtered by passing through cheesecloth and diluted 30 times with distilled water, according to the method of Basra et al. (2011) adapted.

Both macro and micronutrients of MLE were determined using atomic absorption spectrophotometer, according to Silva (2009). In addition, total phenolic was determined by using the Folin-Ciocalteu methodology Barbosa et al. (2019), in which a mix composed of 0.5 mL of MLE, 2.25 mL of Folin-Ciocalteu (7% v/v), 1.75 mL of sodium carbonate, and 0.5 mL of distilled water. Then, the quantification was determined by using a UV-vis spectrophotometer at 765 nm absorbance and the calibration curve was obtained from the gallic acid standard. Similarly, the determination of flavonoid content was performed according to Barbosa et al. (2019), using an aluminum nitrate colorimetric method. In brief, a mix of 0.5 mL of MLE, 0.1 m of aluminium, 0.1 mL of potassium acetate and 4.3 mL of distilled water was prepared and determined in a spectrophotometer at 425 nm absorbance. The standard for the calibration curve was obtained using rutin. Finally, all assays were performed in triplicate.

5.2.3. Germination and seedling growth parameters

Twenty moringa seeds were placed to germinate in water-moistened germinating paper rolls in the amount of 2.5 times the weight of the dried paper. The rolls were packed into germinators at a constant temperature of 25 ° C for 10 days following Pereira et al. (2015). Each treatment was composed of five repetitions and each roll represented a repetition. The following evaluations were performed with the germination test:

- Root protrusion (RP): percentage of seeds with 1 mm long radicle protruded through the seed coat recorded on 10th day after sowing (DAS). The results were expressed in %.
- Germination (G): percentage of normal seedlings recorded on the 10th DAS. The standard adopted to characterize normal and abnormal seedling is shown in Figure 1. A normal seedling was considered when all essential structures were well developed, completed, proportional, and health, according to Brasil (2009). The results were expressed in %.
- First germination count (FGC): percentage of normal seedlings on 5th DAS. The results were expressed in %.
- Root Protrusion Speed Index (RPSI) and Germination Speed Index (GSI): obtained by daily counting of the number of seeds with radicle protruded and the number of normal seedlings observed, respectively, according to the formula proposed by Maguire (1962).
- Mean Germination Time (MGT): calculated by using the number of normal seedlings per day in the equation proposed by Laboriau (1983).
- Shoot and root length of seedling: on the 5th and 10th DAS, 10 seedlings were randomly selected from each roll and manually measured using a graduated ruler to determine the shoot and root length. The results were expressed in cm.seedling^{-1}.
- Vigor and Uniformity index: obtained using seedling length on the 5th and 10th DAS in the equation proposed by Sako et al. (2001).
- Fresh and dry weight of seedling: after measuring the seedling length on the 10th DAS, the fresh weight was determined by using an analytical balance. Then, the seedlings were placed into paper bags, dried into a forced ventilation even at 65 ºC for 48h and weighed in an analytical balance to determine the dry weight. The results were expressed in g.seedling^{-1}.

5.2.3. Estimation of antioxidant enzymes

The activity of antioxidant enzymes catalase (CAT) and ascorbate peroxidase (APX) were determined in primed seeds and unprimed seeds (control). The crude enzymatic extracts were obtained by maceration of 0.2 g of seeds after 16 hours of soaking in 1.5 mL of 50 mM phosphate buffer (pH 7) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (p/v) polyvinylpolypyrrolidone (PVPP) (Peixoto *et al.*, 1999). The homogenized extract was centrifuged at 14,000 g for 20 minutes at 4 °C and the supernatants were used to measure the antioxidant enzymatic activity.

The CAT activity was determined by adding 100 µL of the crude enzymatic of seed extract to 2 mL of reaction medium consisting of 50 mM phosphate buffer (pH 7) and 10 mM H_2O_2. The reduction in absorbance was assessed by spectrophotometer at the wavelength of 240 nm for two minutes reaction, recorded at every 15 seconds (Havir and McHale 1987). The results were expressed in µmol min^{-1}. g^{-1} FW.

The APX activity was determined by adding 100 µL of the crude enzymatic seed extract to 2 mL of reaction medium consisting of 50 mM phosphate buffer (pH 7), 0.1 mM EDTA, 0.25 mM sodium ascorbate and 1 mM H_2O_2. The reduction in absorbance was assessed by spectrophotometer at wavelength 290 nm for 5 minutes, recorded at every 30 seconds (Nakano and Asada 1981). The results were expressed in µmol.min^{-1}.g^{-1} FW.

5.2.4. Plant growth and gas exchange parameters

Hydroprimed seeds and primed seeds with EBL 10^{-8} M, AsA 100 mg.L^{-1}, and MLE 1:30 were sown in 1000 ml polyethylene bags. Non-primed seeds were taken as the control. Three seeds were sown in each bag at a depth of 2 cm using an agricultural substrate. One week later, the more vigorous seedling was left. Each treatment had four repetitions, each one composed of three plants. The plants were kept in a greenhouse for 45 days and the following morphologic parameters were measured, thus plant height using a graduated ruler from the stem base to the last pair of leaves; the stem diameter determined with a digital caliper and the number of leaves by counting fully expanded leaves.

To evaluate gas exchange parameters was used an infrared gas analyzer, IRGA equipment (model LI-6400 xt, Li-color Nebraska, USA). Measurements were taken from mature and fully expanded Moringa leaves from 8:00 to 10:00 AM after 45 DAS. The variables evaluated were the photosynthetic rate (µmolCO_2.m^{-2}.s^{-1}), stomatal conductance (molH_2O.m^{-2}.s^{-1}), transpiration rate (mmolH_2O.m^{-2}.s^{-1}) and internal CO_2 concentration (mol.CO_2.mol.ar^{-1}).

5.2.5. Experimental design and statistical analysis

The experiment was conducted in a completely randomized design (CRD). Germination, seedling growth, and vigor variables were calculated using the Germcalc and PlantCalc functions of the R software (Silva et al., 2019). Data were submitted to normality tests and analysis of variance. The means were compared by the Tukey test at 5% probability using the software R version 4. 2. 1. (R Development Core, 2015).

5.3. Results

5.3.1. Moringa Leaf Extract characterization

The characterization of MLE was performed and the analysis of its constitutes is described as follows: Na (202.50 mg.L^{-1}), K (461 mg.L^{-1}), Ca (214.50 mg.L^{-1}) Mg (101.40 mg.L^{-1}), Fe (0.09 mg.L^{-1}), Mn (0.12 mg.L^{-1}), Zn (0.10 mg.L^{-1}), total phenols (42.10 mg. g^{-1}) and flavonoids (38.17 mg.g^{-1}).

5.3.2 Germination, seedling growth and antioxidant enzymes

Even though the seed germination percentage of *Moringa oleifera* was not affected by seed priming on the 10th DAS (Table 1). The EBL treatment at 10^{-8} M concentration was the most efficient by increasing germination from 33 to 57% on the 5th DAS. Similarly, the EBL 10^{-8} M provided the highest value of GSI. All primed seeds had a reduction in approximately one day of MGT, compared to the control. Contrary to germination percentage, lower values of MGT are positively related to germination in a shorter time. Although none of the seed priming treatments affected the root protrusion percentage, its speed of occurrence increased significantly in primed seeds with EBL 10^{-10} M compared to the control.

The shoot length of seedlings on the 5th DAS increased in all primed treatments, except for hydroprimed seeds and primed seeds with AsA 50 mg.L^{-1} (Table 2). The highest shoot length was observed on primed seeds with AsA 150 mg. L^{-1}. However, only primed seeds with MLE 1:30 performed better than the control evaluated on the 10th DAS, increasing by approximately 1.4 cm of shoot length. In addition, all primed seeds improved the root length of seedlings on the 5th DAS, except for hydroprimed seeds, seeds primed with EBL 10^{-6} M and AsA 50 mg.L^{-1}. These observations of our study suggest that there is a narrow concentration range in which 24-epibrassinolide promotes better seedling growth. The MLE 1:30 showed the highest value the root length of seedlings on the 10th DAS, increasing approximately 4.39 cm over the control.

Seedling uniformity and vigor were also affected by the priming substances applied (Table 3). EBL 10^{-8} M and AsA 100 mg.L^{-1} provided the highest uniformity index on the 5th DAS, although these differences were not significant on the 10th DAS. The vigor index reached the maximum on EBL 10^{-8} M on the 5th DAS. However, MLE 1:30 stood out for being the greater one to improve this parameter on the 10th DAS. The highest value of fresh and dry seedling weight was observed in MLE 1:30 treatment (Table 4).

The antioxidant system was evaluated by the activity of CAT and APX enzymes in unprimed and primed seeds (Table 4). There was no significant difference among priming treatments for APX activity. However, the maximum activity of CAT was under the treatments of AsA 100 and 150 mg.L^{-1}. The lowest values of CAT activity were observed in AsA 50 mg.L^{-1} and EBL 10^{-6} M treatment. Thus, both concentrations were not efficient in improving the effects of seed priming by increasing the antioxidant system activity, which were reflected in the seedling growth, especially the root length of seedlings on the 5th DAS (Table 2).

5.3. Plant growth and gas exchange parameters

Primed seeds with MLE 1:30 was the most efficient to improve the plant height of *Moringa oleifera*, which increased approximately 4.1 cm compared to the control (Fig. 2A). Although the priming products did not show any effect on the stem diameter, the number of leaves was significantly higher in primed seeds with EBL at 10^{-8}M and MLE 1:30 (Fig. 2B and 2C).

The positive effects of seed priming were also observed in the photosynthetic rate of *Moringa oleifera* plants, in which the treatment with MLE 1:30 showed the highest value for this parameter (Fig. 3A). Moreover, treatments with MLE 1:30 and AsA 100 mg.L^{-1} increased significantly the stomatal conductance, compared to the control. Similarly, the concentration of internal CO_2 increased in all products primed seeds, although there was no significant difference in the transpiration rate.

5.4. Discussion

The positive effects of seed priming on plants are recognized for hastening the germination process and improving plant growth under optimal and stressful conditions (Lutts

et al., 2016). Seed priming can reduce imbibition time during germination and also plays an essential role in increasing the speed of germination. Therefore, more vigorous seeds usually take less time to start germination when all external conditions are supplied with water, temperature, and light (Iqbal, 2015). That could be explained by the priming process be consisted of two phases, the first one is partial imbibition followed by re-drying back to the same moisture content before hydration. In fact, this first imbibition provides a "head-start" that accelerate germination (Chen and Arora, 2013; Kubala et al., 2015b). In the present study, all primed seeds increased germination speed, although the most prominent effects were observed in primed seeds with EBL at the concentration of 10^{-10} and 10^{-8} M.

Brassinosteroids are recognized for acting on different processes of plant growth, especially cellular expansion and division (Taiz et al., 2017). Despite being positively regulated by gibberellins during the germination stage, brassinosteroids also contribute to this process but using different metabolic routes (Leubner-metzger, 2001). All plant hormones are characterized by being naturally produced in small quantities. Therefore, BRs are typically known for promoting plant growth in an extreme low amount (Mandava, 1988). Thus, our findings suggested that the 10^{-6} M dose may have interfered in the beneficial effects of BRs to improve germination speed. To maximize the positive effects of BRs, its necessary to adjust its concentration following experimental procedures. For this reason, using exogenous brassinosteroids, such as 24-epibrassinolide seems to be appropriated to improve growth at a certain concentration. Consequently, when the threshold level is reached, the plant may have injurious effects (Wu et al., 2019). In addition, the effects caused by exogenous application of brassinosteroids can vary among different plant species or plant growth stages (Gomes, 2011).

Similarly, the EBL at 10^{-10} and 10^{-8} M improved seedling growth, especially root length at 5 DAS. Reduction in root growth could be associated to one of the first symptoms of seed deterioration during the germination. Even though root protrusion occurs, any failure in this process can affect the root growth for a plant's sensitive part (Bewley et al. 2013). The positive effect of EBL in increasing root growth was reported in sunflower seedlings, in which these both concentrations (10^{-10} and 10^{-8} M) reduced the inhibition of radicle elongation caused by chilling at a rate of 20.6 and 23.9 %, respectively (Gornik and Lahuta 2017). The application of brassinosteroids enhanced the root length under stress conditions such as drought (Fariduddin et al., 2009), lead toxicity (Soares et al., 2020), and saline stress (Larré et al., 2015). In addition, BRs seem to be a nontoxic product and environmentally friendly plant hormone, which has gotten the attention of the scientific community and industry market as a promising technology for enhancing yield and quality of different crops (Kang and Guo 2011).

Overall, the treatment with MLE 1:30 stood out for being the best priming product to improve seedling growth, mainly root and shoot length, vigor index at 10^{th} DAS, and fresh and dry weight. The benefits provided by MLE seems to be linked with the presence of micro and nutrients, such as Na, K, Ca, Mg, Fe, Zn and Mn and non-enzymatic compounds, such as flavonoids and phenolic acids as were described in our study. In addition, *Moringa oleifera* leaves are characterized by the presence of plant hormones such as auxin, gibberellins, cytokinin, salicylic acid, jasmonic acid, and abscisic acid, which could also explain its positive effects on plant growth (Ali et al., 2018; Rehman et al., 2017). The MLE utilization in agriculture is recognized for being practical, non-toxic, and improving agronomic cultures' performance (Rehman et al., 2015; Yasmeen et al., 2013). MLE can be stored for one month at ambient temperature without decreasing its potential effects (Khan et al., 2017). Seed priming treatment with MLE improved seedling length and dry weight of cowpea (Iqbal, 2015) and wheat (Khan et al., 2017). Moreover, MLE applied in the seed priming is also recognized not only for improving plant growth under optimal conditions but also for ameliorating the adverse effects of a variety of stress, such as $HgCl_2$ (Bibi et al., 2016), saline (Nouman et al. 2014; Rady et al. 2013) and drought (Pervez et al., 2017).

Priming technique is based on hydrating seeds until they reach a point where its metabolism is ready for germination germinate. During this process, many biochemical changes occur, such as DNA repair mechanisms, degradation and mobilization of reserves, biosynthesis of proteins, and restoration of membranes. In addition, the enzymatic system is active to deal with the oxidative stress by preventing the generation of reactive oxygen species (ROS) (Marcos-Filho, 2015). It is suggested that seed priming responds to proteins that regulate the oxidative stress on post-priming germination (Kubala et al., 2015a). Some genes, such as CAT2 and PER21, were identified as regulators of antioxidative enzymes, e.g., catalase and peroxidase, respectively, and both operate after the application of seed priming techniques (Kubala et al., 2015a). Under optimal conditions, the cell produces a low amount of free radicals (Mittler, 2002). Although, during the germination process, the antioxidative activity is usually increased to deal with ROS generation to develop the embryonic axis (Garnczarska and Wojtyla, 2008). In the present study, primed seeds with AsA 100 and 150 $mg.L^{-1}$ reached the highest values of CAT activity. It is suggested that improvements caused by AsA treatment applied in seed priming are related to the increase of phenolic activity and the activity of antioxidant enzymes that reflects on the enhancement of seed vigor response (Burguieres et al., 2007). Similarly, in our study we noted the increase of seedling growth (Table 2) and seed vigor parameters (Table 3) in the primed seed with AsA 100 and 150 $mg.L^{-1}$, and probably it consequently reflected on gas exchange parameters such as photosynthetic rate, stomatal conductance, and internal CO_2 concentration of *Moringa oleifera* plants (Fig. 3).

Ascorbic acid improves plant growth by enhancing antioxidant capacity, cell division, and cell enlargement and seems to protect against oxidative damage by controlling cellular redox state (Athar, Khan e Ashraf, 2008). Normally the increase of ROS implies the increase of antioxidative enzymes to minimize the negative effects of oxidative damage in the cell. In okra seeds treated with AsA 100 $mg.L^{-1}$, under conditions of lead stress, the activity of CAT increased significantly (Hussain *et al.*, 2017). Besides ascorbic acid be known as an important part of seed antioxidant defense, its function also includes being a co-substrate of enzyme required to the synthesis of other hormones such as ethylene, gibberellins and abscisic acid. Ascorbic acid plays a role during the whole life span of seeds, from embryogenesis to seed filling and dehydration. It is believed that the seed germinability could be assured by the ability to quickly restore the biosynthesis of enzymes related to the ascorbic acid (De Tullio and Arrigoni, 2003).

Primed seeds often generate plants that show good growth compared to unprimed seeds. However, there is no agreement if that increase in plant growth is provided by a reduction in time to establish the seedling stage or as a result of physiological adjustments induced by seed priming memory (Lutts et al., 2016). In the present study, plants from primed seeds had a higher photosynthetic rate, stomatal conductance, and internal CO_2 concentration (Fig. 3), which may have caused to improvements in morphological parameters such as plant height, steam base diameter, and number of leaves (Fig. 2). The transpiration rate (Fig. 3D) had no significant differences among treatments. Transpiration is important to evaluate the water loss in the plant and correlate with leaf water status. To maintain the plant growth, the transpiration process should not surpass the amount of water absorbed by plant. Otherwise, high values of transpiration rate can indicate that the plant metabolism is under a stressful condition (Mir et al., 2020).

In this study, the MLE was the best product to maximize the results of seed priming technique, mainly related to gas exchange parameters (Fig. 3). Similarly, a previous study with the application of MLE in rocket plants showed to increase not only the photosynthetic rate and stomatal conductance, but also photosynthetic pigments, being correlated to the increase of antioxidative enzymes (Abdalla, 2013). Changes in plant metabolism can increase the photosynthetic CO_2 assimilation, such as improvements in photosynthetic machinery by

electron transport chain and Rubisco activity; higher stomatal efficiency, which can improve water status; or increases in nutritional content i.e. nitrogen uptake (Mohammadi et al., 2017). Seed priming increases the performance of the antioxidative system that reacts against the ROS formation and represents the second line to control the photoinhibition. The detoxification of ROS by antioxidants is an important protection of photosynthetic apparatus (Logan et al., 2006).

Priming benefits are most usually related to plants in non-optimal conditions due to its impact on plant growth be more evident for increasing stress resistance (Lutts et al., 2016). Despite the idea of using seed priming to improve plant growth under optimal conditions is still not well explored, there are some studies focus on agronomical species such as maize (Rehman et al., 2015), wheat (Rehman et al., 2015), and okra (Sharma et al., 2014). However, there is still a lack of information about this subject related to other forest species, especially using plant growth regulators or natural products such as MLE. To sum up, although brassinosteroids have been considered the best product to increase the speed germination of *Moringa oleifera* seeds, MLE at 1:30 stood out as a better one for the general growth of Moringa seedlings and plants.

5.5. Conclusions

The present study showed the beneficial role of seed priming treatment to increase seed germination, seedling, and plant growth of *Moringa oleifera*. Although MLE did not show any positive effect on seed germination, it was the best product used to improve seedling and plant growth for this species, especially for being a natural eco-friendly substance with a low cost.

5.6. References

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Figures and Tables

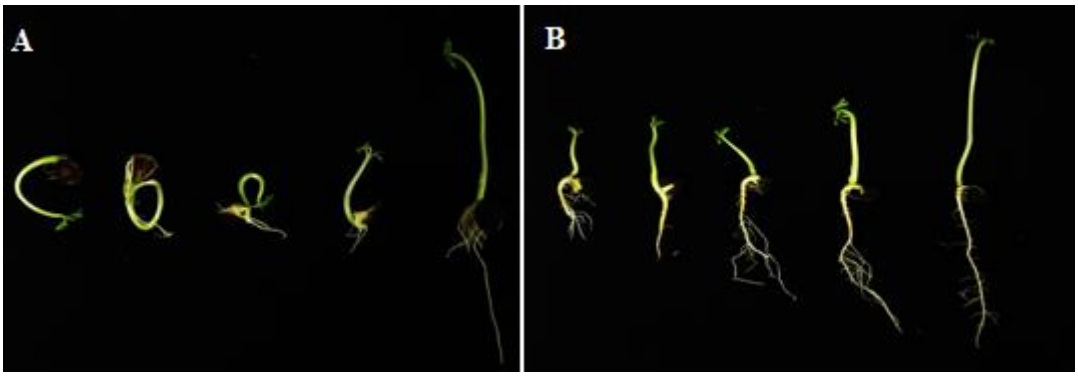

Fig 1. The standard of abnormal (A) and normal (B) *Moringa oleifera* seedlings from the germination test.

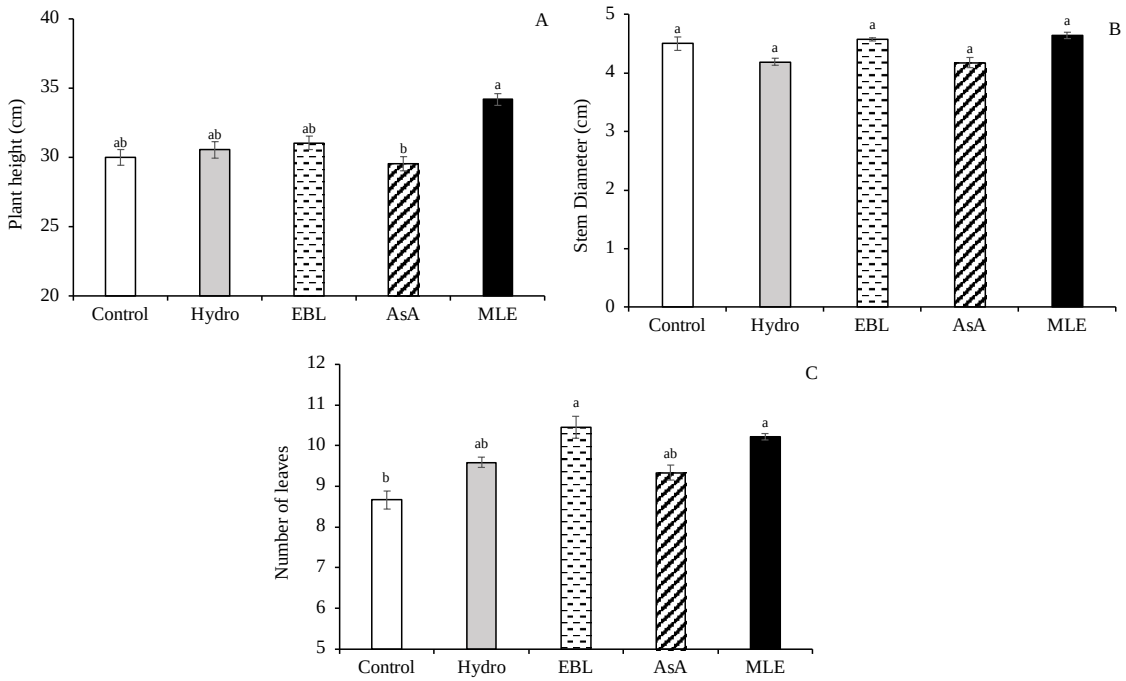

Fig 2. Effect of seed priming on plant height (A), stem base diameter (B) and number of leaves (C) of *Moringa oleifera* seedling grown in greenhouse after 45 days of sowing. Values are means ± SE (n = 4). Control: no priming; Hydro: primed seeds with water; EBL: primed seeds with 24-Epibrassinolide at 10^{-8} M; AsA: primed seeds with AsA at 100 $mg.L^{-1}$; MLE: primed seeds with Moringa Leaf Extract at 1:30.

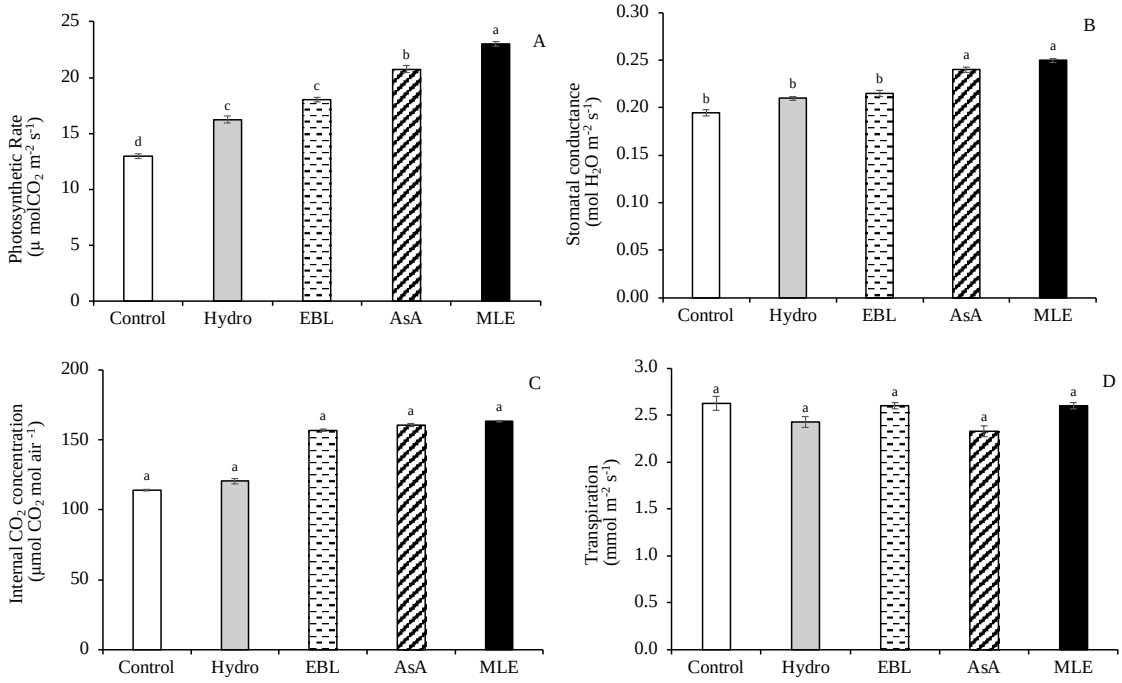

Fig 3. Effect of seed priming on Photosynthetic rate (A), Stomatal conductance (B), Internal CO_2 concentration (C), and Transpiration (D) of *M. oleifera* seedling grown in greenhouse after 45 days of sowing. Values are means ± SE (n = 5) and differences between means were compared by Tukey test (P = 0.05). Means followed by different letters are significantly different. Control: no priming; Hydro: primed seeds with water; EBL: primed seeds with 24-Epibrassinolide at 10^{-8} M; AsA: primed seeds with AsA at 100 $mg.L^{-1}$; MLE: primed seeds with Moringa Leaf Extract at 1:30.

Table 1

Effect of seed priming on Germination (G), First Germination Count (FCG), Germination Speed Index (GSI), Mean Germination Time (MGT), Root Protrusion (RP) and Root Protrusion Speed Index (RPSI) of *M. oleifera*. Values are means ± SE (n = 5) and differences between means were compared by Tukey test (P = 0.05). Means followed by different letters are significantly different.

Priming	Dose	G (%)	FGC (%)	GSI	MGT (days)	RP (%)	RPSI
Non-Primed (Control)	-	47 ± 4.90^{a}	33 ± 7.18^{b}	1.85 ± 0.20^{b}	5.26 ± 0.20^{a}	65 ± 7.58^{a}	5.37 ± 0.62^{c}
Hydro	-	53 ± 6.82^{a}	46 ± 6.96^{ab}	2.49 ± 0.35^{ab}	4.39 ± 0.09^{b}	70 ± 4.18^{a}	7.77 ± 0.77^{abc}
24-EBL	10^{-10} M	57 ± 6.44^{a}	51 ± 6.40^{ab}	2.75 ± 0.30^{ab}	4.29 ± 0.08^{b}	74 ± 3.67^{a}	8.53 ± 0.88^{a}
	10^{-8} M	65 ± 3.54^{a}	57 ± 5.39^{a}	3.11 ± 0.20^{a}	4.37 ± 0.11^{b}	72 ± 3.39^{a}	8.22 ± 0.19^{ab}
	10^{-6} M	45 ± 4.06^{a}	35 ± 2.92^{ab}	2.14 ± 0.23^{ab}	4.42 ± 0.18^{b}	70 ± 8.72^{a}	6.36 ± 0.79^{abc}
AsA	50 $mg.L^{-1}$	50 ± 4.74^{a}	39 ± 5.57^{ab}	2.18 ± 0.24^{ab}	4.78 ± 0.13^{ab}	76 ± 5.34^{a}	6.38 ± 0.49^{abc}
	100 $mg.L^{-1}$	60 ± 2.74^{a}	50 ± 2.74^{ab}	2.73 ± 0.12^{ab}	4.51 ± 0.06^{b}	77 ± 3.00^{a}	7.07 ± 0.26^{abc}
	150 $mg.L^{-1}$	49 ± 2.92^{a}	42 ± 2.00^{ab}	2.35 ± 0.09^{ab}	4.34 ± 0.24^{b}	62 ± 4.64^{a}	6.25 ± 0.32^{abc}

MLE	1:30	57 ± 4.06^{a}	45 ± 3.54^{ab}	2.53 ± 0.17^{ab}	4.67 ± 0.17^{ab}	70 ± 4.47^{a}	5.67 ± 0.34^{bc}

Table 2

Effect of seed priming on shoot and root length of *M. oleifera* seedlings on 5th and 10th day after sowing. Values are means ± SE (n = 5) and differences between means were compared by Tukey's test (P = 0.05). Mean followed by different letters are significantly different.

Priming	Dose	Shoot Length (cm.seedling^{-1})		Root Length (cm.seedling^{-1})	
		5th day	10th day	5th day	10th day
Non-Primed (Control)	-	1.45 ± 0.08^{c}	4.62 ± 0.27^{b}	4.53 ± 0.63^{b}	6.59 ± 0.63^{b}
Hydro	-	1.96 ± 0.11^{bc}	4.59 ± 0.19^{b}	5.64 ± 0.56 ^{ab}	6.78 ± 0.56^{b}
	10^{-10} M	2.23 ± 0.06^{ab}	4.80 ± 0.26^{ab}	7.34 ± 1.10^{a}	8.57 ± 1.10^{ab}
24-EBL	10^{-8} M	2.43 ± 0.08^{ab}	5.06 ± 0.11^{b}	7.57 ± 0.70^{a}	8.10 ± 0.70^{ab}
	10^{-6} M	2.14 ± 0.14^{ab}	4.36 ± 0.26^{b}	5.70 ± 0.73^{ab}	7.49 ± 0.73^{b}
	50 mg.L^{-1}	1.91 ± 0.16^{bc}	4.90 ± 0.12^{b}	6.65 ± 0.81^{ab}	9.39 ± 0.81^{ab}
AsA	100 mg.L^{-1}	2.18 ± 0.07^{ab}	4.80 ± 0.17^{b}	7.29 ± 0.80^{a}	9.21 ± 0.80^{ab}
	150 mg.L^{-1}	2.75 ± 0.26^{a}	4.79 ± 0.19^{b}	6.82 ± 0.87^{a}	7.46 ± 0.87^{ab}
MLE	1:30	2.32 ± 0.12^{ab}	6.03 ± 0.28^{a}	7.20 ± 0.98^{a}	10.98 ± 0.98^{a}

Table 3

Effect of seed priming on Uniformity and Vigor Index of *M. oleifera* seedlings on 5th and 10th day after sowing. Values are means ± SE (n = 5) and differences between means were compared by Tukey test (P = 0.05). Mean followed by different letters are significantly different.

Priming	Dose	Uniformity Index		Vigor Index	
		5th day	10th day	5th day	10th day
Non-Primed (Control)	-	649 ± 29^{b}	710 ± 17^{a}	490 ± 17^{c}	660 ± 35^{b}
Hydro	-	683 ± 52^{ab}	712 ± 28^{a}	574 ± 28^{bc}	673 ± 32^{b}
	10^{-10} M	780 ± 30^{ab}	735 ± 44^{a}	712 ± 44^{ab}	794 ± 71^{ab}
24-EBL	10^{-8} M	817 ± 15^{a}	798 ± 22^{a}	739 ± 22^{a}	785 ± 43^{ab}
	10^{-6} M	697 ± 41^{ab}	691 ± 38^{a}	583 ± 38^{bc}	723 ± 52^{b}
	50 mg.L^{-1}	728 ± 45^{ab}	758 ± 47^{a}	650 ± 47^{abc}	853± 55^{ab}
AsA	100 mg.L^{-1}	816 ± 29^{a}	819 ± 31^{a}	719 ± 31^{ab}	859 ± 44^{ab}
	150 mg.L^{-1}	721 ± 28^{ab}	771 ± 33^{a}	665 ± 33^{ab}	734± 51^{ab}
MLE	1:30	783 ± 25^{ab}	735 ± 37^{a}	704 ± 37^{ab}	954 ± 72^{a}

Table 4

Effect of seed priming on fresh and dry weight of *Moringa oleifera* seedlings and the activity of CAT and APX enzymes in *M. oleifera* seeds. Values are means ± SE (n = 5) and differences between means were compared by Tukey test (P = 0.05). Mean followed by different letters are significantly different.

Priming	Dose	Fresh Weight	Dry Weight	CAT activity	APX activity
		(g.seedling^{-1})		Mmol. min^{-1} g FW	
Non-Primed (Control)	-	0.274 ± 0.017^{b}	0.022 ± 0.001^{b}	0.06 ± 0.01^{ab}	0.22 ± 0.06^{a}
Hydro	-	0.259 ± 0.017^{b}	0.020 ± 0.002^{b}	0.14 ± 0.05^{ab}	0.27 ± 0.09^{a}
24-EBL	10^{-10} M	0.241 ± 0.012^{b}	0.020 ± 0.002^{b}	0.13 ± 0.06^{ab}	0.47 ± 0.21^{a}
	10^{-8} M	0.263 ± 0.011^{b}	0.023 ± 0.002^{ab}	0.05 ± 0.03^{ab}	0.82 ± 0.24^{a}
	10^{-6} M	0.240 ± 0.007^{b}	0.017 ± 0.001^{b}	0.02 ± 0.01^{b}	0.53 ± 0.31^{a}
AsA	50 mg.L^{-1}	0.265 ± 0.016^{b}	0.021 ± 0.001^{b}	0.02 ± 0.01^{b}	0.05 ± 0.02^{a}
	100 mg.L^{-1}	0.282 ± 0.008^{b}	0.022 ± 0.003^{b}	0.20 ± 0.02^{a}	0.05 ± 0.01^{a}
	150 mg.L^{-1}	0.250 ± 0.007^{b}	0.019 ± 0.001^{b}	0.19 ± 0.03^{a}	0.22 ± 0.05^{a}
MLE	1:30	0.391 ± 0.026^{a}	0.033 ± 0.005^{a}	0.11 ± 0.04^{ab}	0.40 ± 0.18^{a}

6. MANUSCRIPT 3
CHILLING AND FREEZING STRESS TOLERANCE IN *MORINGA OLEIFERA* LAM.

Artigo aceito no periódico Scientia Horticulturae

ABSTRACT
Chilling and freezing stress tolerance in *Moringa oleifera* Lam.

Moringa oleifera Lam. is a tree species and popularly known for its multiple uses. The high nutrional value of moringa leaves make it a valuable product for human and animal nutrition. Native of India, moringa grows well in tropical and subtropical areas, being spread worldwide. However, the cold tolerance for this species remains unclear. This study aimed to evaluate the chilling tolerance of moringa under controlled low-temperature regimes and duration and assess whether this species has freeze-tolerance and cold acclimation ability. To evaluate chilling stress, we used three temperature regimes: 20/15, 15/10, and 10/5 °C, of which 10/5 °C was chosen to further investigate the effects of duration and recovery. Morphological (seedling height, number of leaves, number of leaflets, fresh weight, dry weight, and leaf area) and physiological indicators such as a chlorophyll fluorescence parameter (Fv/Fm) and electrolyte leakage (%) were used. All physiological parameters were compromised at progressively cooler temperature regimes. Chilling injury to photosynthetic apparatus and cellular membranes at 10/5 °C for 4 days was completely reserved during the recovery period, In contrast, 8 days of stress caused more servere injury which was only partially recoverable, and 12 days of stress inflicted severest and irreversible injury.The freezing tolerance and cold acclimation ability were evaluated using a laboratory freeze-thaw protocol. The tissues were 50% injured (LT_{50}) at -2.8 °C. In addition, our data indicated the moringa did not show cold acclimation ability after 4 days at 15/10 °C or 4 days at 10/5 °C. The cold tolerance in moringa appears to be a complex system regulated by intensity and duration, and this study provided the baseline information to understand the physiological mechanism involved in these processes.

Key-words: cold tolerance; stress duration; recovery; chlorophyll fluorescence; F_v/F_m; LT_{50}; cold acclimation

6.1. Introduction

The natural distribution of plants is deeply regulated by the low and high-temperature ranges. Therefore, plants have different degrees of sensitivity to cold which can be affected by altitudes, distance from coastal areas, and latitudes (Nilsen and Orcutt, 1996). Cold tolerance is the ability of plants not only to survive under low temperatures but also without limiting their growth. Chilling-sensitive species normally show injury upon exposure to low but non-freezing temperatures, typically ranging from 10 to 25 °C (Raison and Lyons, 1986). In contrast, freezing stress is caused by temperatures below 0 °C resulting in ice crystallization in the plant tissue. However, plants can increase their freezing tolerance when exposed to non-injurious cold for certain duration (Levitt, 1980). Cold acclimation is a process which involves cellular, physiological, morphological, and biochemical reprogramming that induces improved freezing tolerance (Levitt, 1980). Tropical plants are recognized for being both chilling sensitive as well as lacking cold acclimation ability (Maleki and Ghorbanpour, 2018).

Moringa oleifera Lam., a member of *Moringaceae* family, is popularly known as the drumstick or miracle tree, and is recognized for its various beneficial uses. Moringa leaves are a great source of protein, minerals (Ca, P, Mg, Na, K, Fe, Mn, Zn), vitamins (A, B1, B2, B3, C, and E), carotenoids, phenols, and flavonoids. The seeds are rich in oil that can be used as food component as well as in the cosmetic industry and as a potential biofuel (Foidl et al., 2001; Jain et al., 2020). Additionally, moringa seed extract has coagulant properties with potential application in water purification systems (Ghebremichael et al., 2005). Finally, moringa is also considered as a medicinal plant due to its several bioactive compounds with anti-inflammatory, antibacterial, and antioxidative properties (Singh et al., 2020).

Moringa is native to Northern India (Himalayan foothills) and grows well in different tropical and subtropical areas, especially Africa, Asia, and South America (Leone et al., 2015). In Brazil, moringa was introduced as an ornamental tree and is well adapted to Northeast climatic conditions. However, its initial growth seems to be impaired when it cultivated in South areas of Brazil during the winter (Costa et al., 2015). The optimal growth temperature for moringa has been suggested as 25–35 °C, typical of many chilling-sensitive crops (Trigo et al., 2020). Therefore, low temperature is considered one of the most limiting factors to potential geographical expansion of moringa cultivation into colder regions (Godino et al., 2017). However, very limited information is available about the effects of low temperature on moringa growth. In addition, there is no report of a systematic study using controlled temperature regimes to test chilling tolerance of this species. Moreover, no scientific investigation exists, to date, of whether moringa can tolerate freezing or presence of ice in its tissues. The objective of this study was to: (1) evaluate the chilling tolerance of *Moringa oleifera* Lam. under controlled low-temperature regimes varying in both the intensity (degree) as well as the duration, and (2) assess whether *Moringa oleifera* Lam. has any freeze-tolerance and cold acclimation ability.

6.2. Material and Methods

6.2.1. Plant material and growth conditions

Seeds of *Moringa oleifera* Lam. from the provenance of Croix-des-Bouquets, Haiti (18° 34′ 34″ N, 72° 13′ 37″ W) were obtained from a commercial supplier (Moringa farms, FL, USA). The seeds were soaked overnight and sown in plastic pots with Sunshine LC-1 mix substrate (Seba Beach, Alberta, Canada). Optimum germination temperature for moringa seeds has been reported to be 20-30 °C (Muhl et al., 2011). Accordingly, moringa seeds were germinated under greenhouse conditions with minimum, maximum, and average daily temperatures of 20.4, 26.8, and 22.4 °C, respectively (Fig. 1). The photosynthetically active radiation (PAR) at seedling height was measured at 8:30 am (~270 μmol m^{-2} s^{-1}), 12 pm (~620 μmol m^{-2} s^{-1}), and 4:30 pm (~150 μmol m^{-2} s^{-1}). Supplemental lighting was provided by 208 W Sun System III lamps (Sunlight Supply Inc, Vancouver, WA, USA) from 8 am to 11 am and from 3 pm to 6 pm. The seedlings were watered as needed and fertigated at 13 day-old with 300 ppm EXCEL nutrient solution (Scotts Sierra Horticultural Products Company, Marysville, OH). At 16 days old, seedlings were transferred to growth chambers where the low-temperature treatments were applied and used in the trials described below. At 19 days old, the second fertigation was applied both in seedlings that were in the chambers and in the greenhouse.

6.2.2. Estimation of chilling tolerance

6.2.2.1. Trial 1: Low-temperature regimes

Seedlings were placed into three separate growth chambers (Percival Scientific, Inc.) maintained at 20/15 °C, 15/10 °C, and 10/5 °C (D/N) with 11-h photosynthetically active radiation (PAR) (~250 μmol m^{-2} s^{-1}). Plants were exposed to these temperatures for 7- and 14-day stress (DS) treatments, labeled as 7DS and 14DS. Then, they were transferred back to the greenhouse to recover for 1 d (1DR) or 3 d (3DR). The unstressed control (UNS-control) was kept in the greenhouse during the whole trial. A total of 32 plants were used for this experiment, with each of the 8-sample group (temperature x duration x recovery treatment plus the control) comprising 4 seedlings. The experiment was conducted in a completely randomized design (CRD). Data were submitted to normality tests and analysis of variance. The means were compared by the Dunnett test at 5% probability using the software R version 4. 2. 1. (R Core Team, 2022).

6.2.2.2. Trial 2: Low-temperature duration

Based on the results from Trial 1, 10/5 °C (D/N) stress level was selected to investigate the effect of this stress level on seedlings for three durations, 4 d (4DS), 8 d (8DS), and 12 d (12 DS). Stressed seedlings were returned to greenhouse for a 4 d recovery period (4DR). The UNS-control treatment was kept in the greenhouse during the whole trial. A total of 30 plants were used, which each of the 6-sample group (duration x recovery treatment plus the control) comprising 5 seedlings. The experiment was conducted in a completely randomized design (CRD). Data were submitted to normality tests and analysis of variance. The means were compared by the Student's *t*-test at 5% probability using the software R version 4. 2. 1. (R Core Team, 2022).

6.2.2.3. Seedling and leaf growth measurements

Seedling growth was evaluated by measuring seedling height using a graduated ruler and counting the number of compound leaves and leaflets. Leaf growth was estimated by measuring fresh weight (FW), dry weight (DW), and leaf area (LA). First, all leaves of each seedling from each treatment were weighed to determine the FW. Next, the same leaves were immediately measured for leaf area using LI-3100 Area Meter (LI-COR, Inc., Lincoln, NE, USA). Finally, the DW of the same leaves was measured after being dried in an oven at 65 °C for 72 h.

6.2.2.4. Chlorophyll fluorescence

Chlorophyll fluorescence (F_v/F_m; indicator of the maximum quantum yield efficiency of PSII) was measured using the Handy-PEA fluorometer (Hansatech Instruments Ltd, King's Lynn, UK). Each measurement was taken on the terminal leaflet of the first fully expanded leaf of each seedling. Four seedlings of each treatment were used for the measurements. The leaflets were dark-adapted for ~30 min using leaf clips. Maximum fluorescence was induced by a light pulse intensity at 3000 μmol m^{-2} s^{-1} for 1s.

6.2.2.5. Electrolyte leakage

One leaflet per seedling from each treatment, after the stress or recovery treatments, was placed in a test tube containing 20 ml of deionized water and placed into vacuum infiltration (3 min each at ~70 kPa). The samples were shaken at 200 rpm for 2h. A conductivity meter (model 3100; YSI Inc., Yellow Spring, OH) was used to determine the initial electrolyte leakage. Then, the samples were autoclaved, and the final electrolyte leakage was measured when they reached room temperature (~20 °C). The percent electrolyte leakage was calculated by the formula: (Initial Leakage/Final Leakage) x 100.

6.2.3. Estimation of leaf freezing tolerance and cold acclimation

A temperature-controlled freeze-thaw protocol (Min et al., 2014) employing a glycol bath (Isotemp 3028; Fisher Scientific, Pittsburgh, PA, USA) was used to test leaflet freezing tolerance of 35-day-old seedlings. Petiolate leaflets were placed in a 2.5 x 20 cm test tube with 100 μl of deionized water and slowly cooled (0.5 °C/30 min) from 0°C down to -6°C. Ice nucleation in the leaf tissues was performed at -1.0 °C by dropping small ice crystals in the test tubes. The leaflets were held for ~30 min at each temperature and thawed on ice overnight. Unfrozen control (UFC) leaflets were kept at 0 °C during the whole freeze-thaw cycle. Then, samples were removed from ice and kept in the refrigerator at 4 °C for 1h, followed by 1h at room temperature (~20 °C). Thawed samples were processed for ion-leakage measurements as described under section 2.2.5. Percent injury values were calculated from the ion-leakage data as described by Lim et al. (1998). LT_{50}, the temperature causing 50% injury, was defined as the leaflet freezing tolerance. For this experiment, leaflets from seven plants were randomly

distributed in the following temperature treatments: Unfrozen control (UFC), -0.5, -1.0, -1.5, -2.0, -2.5, -3.0, -3.5, -4.0, -4.5, -5.0, -5.5, and -6.0 °C. Each treatment was composed of five technical replications (one leaflet per tube). This experiment was repeated twice.

Cold acclimation ability of moringa seedlings was evaluated after non-acclimated (NA) plants (those maintained under greenhouse conditions) were exposed to two cold acclimation regimes, i.e. 15/10 °C for 4-d or 10/5 °C for 4-d. Thereafter, leaflet tissues were evaluated for freezing tolerance as described above. The leaflets from seven plants were randomly distributed in each temperature treatment, each including five technical replications (one leaflet per tube).

6.3. Results

6.3.1. Chilling tolerance

6.3.1.1. Low-temperature regimes

After 7-day stress (7DS), the seedling height was significantly reduced in all temperature regimes evaluated, compared to the UNS-control (Fig. 2). After 14-day stress (14DS), the seedling height decreased by 2.8 cm (20/15 °C and 15/10 °C treatments) and 4.5 cm (10/5 °C treatment). Similarly, the number of leaves and leaflets decreased significantly after 7DS in all low-temperature regimes evaluated. At 14DS, such reduction was 33, 58, and 58 % in the leaf number, and 54, 77, and 80 % in the leaflet number at 20/15 °C, 15/10 °C, and 10/5 °C respectively, compared to UNS-control.

Based on the F_v/F_m results, 20/15 °C stress was not significantly different from UNS-control (0.840; Fig. 3) after 7DS. However, F_v/F_m was reduced to 0.517 and 0.191 at 15/10 °C and 10/5 °C treatments, respectively; after one day of recovery (7DS+1DR), both treatments almost fully recovered (~0.726). Similarly, 20/15 °C stress had no significant difference in F_v/F_m from UNS-control at 14DS, whereas 15/10 °C and 10/5 °C treatments were significantly reduced from 0.832 (UNS-control) to 0.564 and 0.102, respectively. However, after three days of recovery (14DS+3DR), no recovery of F_v/F_m was noted for 10/5 °C treatment. Therefore, exposure to 10/5 °C for 14 d was adjudged as irreversible stress, and therefore plant response to '10/5 °C stress level' was further investigated for shorter (than 14-d) durations in trial 2 (below).

6.3.1.2. Low-temperature durations at 10/5 °C stress

All three durations (4d, 8d or 12d) at 10/5 °C stress level i.e., 4DS, 8DS, and 12DS, significantly reduced the seedling height, number of leaves, and number of leaflets, compared to UNS-Control (Fig. 4 A, B, C). None of these parameters recovered even after 4-day of recovery (-4DR). On the contrary, the leaf and leaflet numbers even worsened during the 'recovery' period when stressed for 8 d or 12 d. Similarly, while a slight recovery was noted in the FW, DW and leaf area during a 4-d recovery (-4DR) following a 4-d stress, the reduced values for these parameters at 8 d-stress and 12-d stress indeed worsened further during the 4-d recovery period (4DR; Fig. 5).

The maximum quantum yield efficiency of PSII *(F_v/F_m)* was significantly decreased from 0.827 (UNS-Control) to 0.564 after 4DS (Fig. 6) at 10/5 °C. However, the complete recovery occurred after 4 days under greenhouse conditions. After 8DS, F_v/F_m decreased to 0.207, but the full recovery did not occur after 4-day under greenhouse conditions (recovered samples had F_v/F_m of 0.751 compared to 0.837 in UNS-control). After 12DS, F_v/F_m decreased to 0.148, and the seedlings did not show any sign of recovery after 4-day; on the contrary, F_v/F_m further reduced to 0.031.

The electrolyte leakage increased from 3% in UNS-control to 10 % after 4-day chilling stress (Fig. 7). After a 4-day recovery, the leakage decreased to 3%, hence the tissues

completely recovered. After 8-day stress, the leakage increased to 22 % compared to 3% in UNS-control. However, the partial recovery happened after 4-day in greenhouse conditions, reaching the leakage of ~5 %. On the other hand, the leakage was 38 % after 12-day stress and increased further to 59% during the 4-day recovery period. The visual symptoms of chilling injury/stress are shown in Fig. 8.

6.3.2. Freezing tolerance and cold acclimation

The freeze-thaw response curve for *Moringa oleifera* leaf tissues is shown in Fig. 9. The non-acclimated leaflets did not show any injury when frozen up to -1.5 °C. An exceedingly low injury (based on enhanced electrolyte leakage) was first noted at -2 °C (only 1 % injury). Thereafter, it increased progressively at colder temperatures reaching at ~90% at -6 °C, the coldest sub-freezing temperature used in this study. A sharp increase in freeze-injury during this sigmoid response was noticed between -2 °C and -3.5 °C. The LT_{50} for moringa leaflets was estimated to be -2.8 °C corresponding to the mid-point of injury progression.

The LT_{50} for acclimated leaflets at 15/10 °C for 4 days was ~ -2.8 °C, similar to non-acclimated leaflets (Fig. 9). However, the injury at -3 to -3.5°C were lower (~ 40-70%) for acclimated tissues compared to the non-acclimated ones (~75-90%). The 4-day acclimation at 10/5 °C also did not result in any gain in freezing tolerance (defined as LT_{50}). Moreover, while the freeze-injury (% leakage) was lower up to -2 °C in these cold acclimated tissues (compared to acclimation at 15/10 °C), the injury increased more steeply between -2.5 and -3°C (Fig. 9).

6.4. Discussion

6.4.1. Chilling Tolerance

6.4.1.1. Trial 1: Degree of chilling

Few studies have reported the effects of low temperature on *Moringa oleifera* Lam. growth (Muhl et al., 2011; Costa et al., 2015). One study (Costa et al., 2015) evaluated the initial growth of moringa plants cultivated under field conditions in the South region of Brazil during the autumn/winter season, which has a subtropical climate and reaches approximately 17 °C during the coldest month. The authors reported increased plant mortality (~60% after 60 days) under these conditions. In addition, the height of moringa plants was approximately 7.3 cm after 30 days of emergence with only slight increase of 1.4 cm after another 30-d. In our study, moringa seedlings were nearly two times taller (14.3 cm; Fig. 2) when 30-day-old under greenhouse conditions. Clearly, such difference can be attributed to the higher average temperature in the greenhouse (~ 22 °C), supposedly more favorable to moringa growth.

In another study by Muhl et al. (2011), effects of three low-temperature regimes (30/20 °C, 25/15 °C, and 20/10 °C) (D/N) on seed germination and seedling growth were evaluated for 30 days after sowing. These authors reported that seedlings height reduced by approximately 125 % and 200% at 25/15 °C and 20/10 °C, respectively, compared to seedlings at 30/20 °C (considered the most favorable treatment regime). In our study, the seedling height was reduced by 24 % at 20/15 °C and 15/10 °C, and 46 % at 10/5 °C, compared to UNS-control when the seedlings were at the age of 30-day old (i.e., after a 14-day stress). A significantly smaller reduction in seedling height after chilling stress in the present study compared to that reported by Muhl et al. (2011), could be attributed to the following: Muhl et al. (2011) germinated the seeds under low-temperature regimes with the entire duration of low-temperature treatment of 30-d, whereas, in our study, moringa seedlings had only 14 days of stress at respective low-temperature regimes.

None of these previously published studies reported the effects of low-temperature regimes on the number of leaves or leaflets. Moringa leaf production is an important parameter for this species due to their value as a source for vitamins, carotenoids, flavonoids, and phenolic

acids used for human and animal nutrition, and, therefore, effects of low temperatures on leaf growth also needs attention (Leone et al., 2015). Our results indicate that all low-temperature regimes significantly reduced the leaf and leaflet number, even at 20/15 °C.

Chilling stress impairs plant growth due to the damage caused to photosynthetic processes. When exposed to cold temperatures, the plants adapted to warmer climates suffer dysfunctions in the thylakoid membrane, the site photochemical and electron transport reactions (Allen and Ort, 2001). One of the methods to assess the health of photosynthetic apparatus is to measure chlorophyll fluorescence, a reflection on PSII efficiency (Hassannejad et al., 2020; Horaczek et al., 2020). The F_v/F_m parameter, represented by the ratio of variable (F_v) and maximum (F_m) fluorescence, indicates the maximum photochemical quantum yield of photosystem II. This parameter has been widely used in chilling stress studies with different species such as *Vitis vinifera* L. (Aazami et al., 2021), sweet potato (Lin et al., 2007), *Medicago sativa* (Lang et al., 2020), and *Paphiopedilum* species (Yang et al., 2017). In the present study, the F_v/F_m parameter was a remarkable stress indicator to define a threshold of low-temperature tolerance for moringa (Fig. 3). In addition, the evaluation of recovery was extremely important to define this limit, in which 1-day recovery after 7-day stress was able to completely restore the PSII efficiency in all low-temperature regimes evaluated. However, when the duration of the stress increased to 14 days, even 3 days of recovery was not sufficient to restore this parameter at 10/5 °C. These observations highlight the importance of an exposure of chill-stressed plants to posterior optimum conditions for certain duration to fully evaluate the final injury response.

6.4.1.2. Trial 2: 'Stress dose - degree x duration'

The visual symptoms of chilling stress under different durations at the defined critical temperature regime (10/5 °C) in the moringa seedlings are shown in Fig. 8. The first symptom visualized at 4DS was the leaf wilting in seedlings. Such rapid wilting is known to be a common symptom of chilling stress (Allen and Ort, 2001). However, the intensity of leaf wilting after having been exposed for another 4 days or 8 days to the same chilling temperature appears somewhat milder than at 4DS. We propose such discrepancy to be a result of 'cold shock' experienced by seedlings earlier in this timeline manifested in reduced hydraulic conductivity. Whereas such loss of water conductance in xylem might have been somewhat recovered over time. It is important to note, however, that despite an apparent less leaf wilting at 8DS and 12 DS compared to 4DS, the seedlings after former two chilling durations were more stressed, as evident by the relative loss in FW, DW, and LA (Fig. 5), reduction in Fv/Fm (Fig. 6), and enhanced membrane injury / ion-leakage (Fig. 7). This clearly points to the effect of chilling 'dose', i.e. the combined effect of 'degree' and 'duration'. That seedlings were more severely stressed at 8DS and 12DS than at 4DS was further substantiated by the data for these stress indicators (growth, F_v/F_m, and ion-leakage) during the 4-day recovery (4DR). Our data indicate that when allowed to recover in the greenhouse under optimum temperature for 4 days exhibited substantial recovery across all stress indicators, along with a regain of leaf turgor (Fig. 5, 6, 7, 8). Recovery for seedlings stressed for 8 days (8DS), however, was relatively milder as well as sporadic; for example, significant recovery was noted for PSII efficiency (F_v/F_m) but plant growth and membrane damage mostly remained unrecovered. Ostensibly, plants exposed for 12 days of chilling stress (12DS) were irreversibly injured as none of the stress parameters recovered during the 4DR. It has been suggested that if the stress duration is too long, when the plant tissue is rewarmed, the injury and cellular degradation are accelerated (Nilsen and Orcutt, 1996).

Curiously, while 8DS+4DR seedlings exhibited substantial chilling injury (chlorosis) their PSII efficiency (F_v/F_m) does not seem to reflect such an intense injury. A clear explanation for this apparent contradiction is unclear. However, it may be related with the procedural limitations of measuring chlorophyll fluorescence. An individual leaflet as well as the area on

the leaflets used for placing clamps might have been much less chlorotic. Leaves and individual leaflets did not sustain uniform chlorosis during the recovery period.

6.4.2. Freezing tolerance and cold acclimation

The electrolyte leakage assay of freeze-thaw stressed tissues has been widely used and accepted method to evaluate freezing tolerance in plants. This method is based on conductivity measurements to assess the integrity of plasma membranes (Thalhammer et al., 2020). The degree of freezing tolerance is usually expressed by an index of ion leakage, the LT_{50}, and determines the subzero temperature at which 50% of injury is caused (Lim et al., 1998). However, there is no report in literature to date about the freezing tolerance for *Moringa oleifera.* The present study presents the first attempt to test moringa can tolerate presence of ice in its leaf tissues, and if so, does it have any ability to increase its freezing tolerance when exposed to cold acclimating environment.

Our data indicate that *Moringa oleifera* leaflets can tolerate ice formation in its tissues and has an LT_{50} of - 2.8 °C (Fig. 9A). Our freezing protocol includes 'ice-nucleation' step at relatively milder sub-zero temperature to ensure that all leaf samples under investigation indeed froze extracellularly (Arora, 2018). Though a surprising response by a tropical species, this result is consistent with a prior report using another tropical tree species, *Pisidium guajava* L., leaves of which were shown to be freeze-tolerant with an LT_{50} of -2.5 °C (Hao et al., 2009).

The ability to cope with freezing temperatures can also be dependent on the seed source (Haase, 2011). The seeds used in this study are from Croix-des-Bouquets' provenance, Haiti, where the average temperature in the coldest month is ~18 °C. A question may arise if moringa seeds from a colder provenance might show even higher freezing tolerance and deserves further investigation.

Surprisingly, our study also revealed that *Moringa oleifera* can tolerate up to - 2 °C without causing substantial injury to plasm membrane integrity (Fig. 9A). It is noteworthy, however, that the freezing protocol used to evaluate the freezing tolerance of moringa leaves involves holding the frozen tissue at a sub-freezing temperature for only ~ 30 min, followed by a thaw-rehydration at 0-4 °C for ~12 h. Therefore, this protocol evaluates effects only in a short-term freezing whereas prolonged freezing duration could cause more severe injury.

One mechanism to increase the freezing tolerance in plants is gradual exposure to low and nonfreezing temperatures to provide cold acclimation. This process provokes physiological adjustments to reduce the injury caused to the cell membranes during a freezing event (Thomashow, 1999). To provide a proper acclimation process, the temperature and duration need to be well adjusted. In our study, we chose temperature regimes of 15/10 and 10/5 °C to test the cold acclimation in moringa seedlings, the temperature regimes which were previously used to assess the chilling tolerance.

Based on LT_{50} values, moringa leaflets did not exhibit cold acclimation ability at 15/10 °C for 4 days (LT_{50} -2.8 °C; Fig. 9 B). Moreover, reducing the temperature regime to 10/5 °C for 4 days also did not increase freezing tolerance (Fig. 9 C).

6.5. Conclusions

The results presented here provide baseline information on the cold tolerance of *Moringa oleifera* Lam. Our study demonstrated that low-temperature stress is a key factor dependent on intensity and duration. The temperature regime of 10/5 °C caused a reduction in growth and photosynthetic capacity in moringa. A stress duration of 8 days caused an unrecoverable injury manifested by lack of adjustments in cell membrane integrity and in repair of chlorophyll fluorescence indicators. We also have shown, by using a laboratory freeze-thaw test, that *Moringa oleifera* provenance used in this study has freezing tolerance with an LT_{50} of -2.8 °C. But this species does not have cold acclimation ability. More studies are warranted to

provide an in-depth investigation of cold tolerance in a range of moringa varieties / cultivars across diverse provenances. This can be the next step to improving cold tolerance of this species via breeding programs.

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Figuras e Tabelas

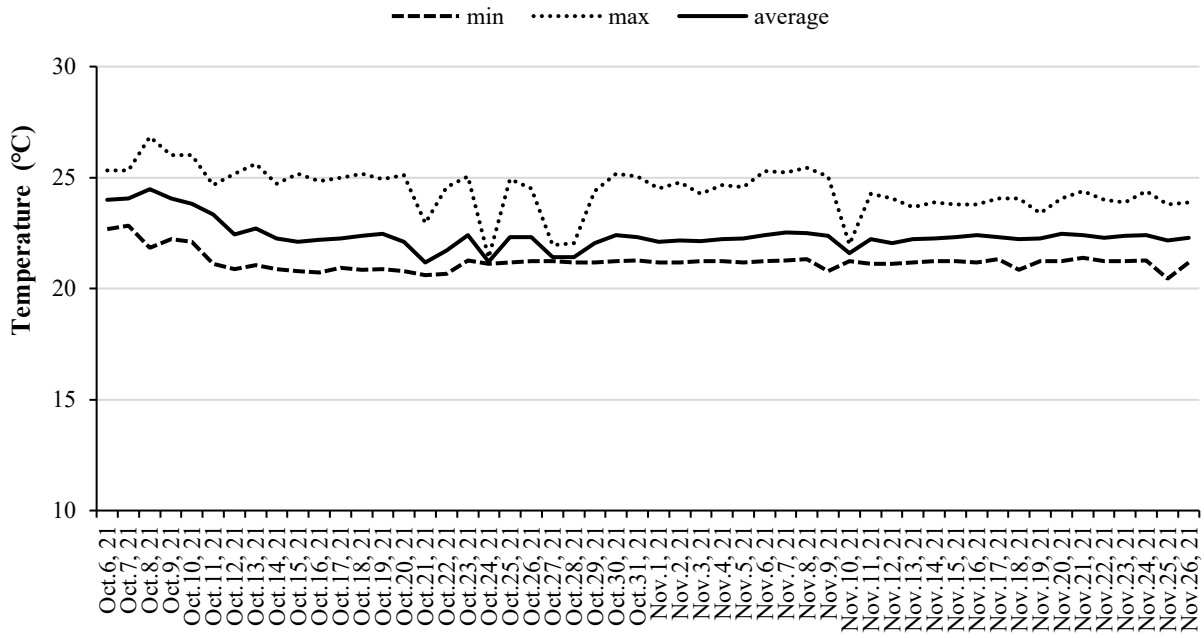

Figure 1: Minimum (min), maximum (max), and average daily temperatures in the greenhouse.

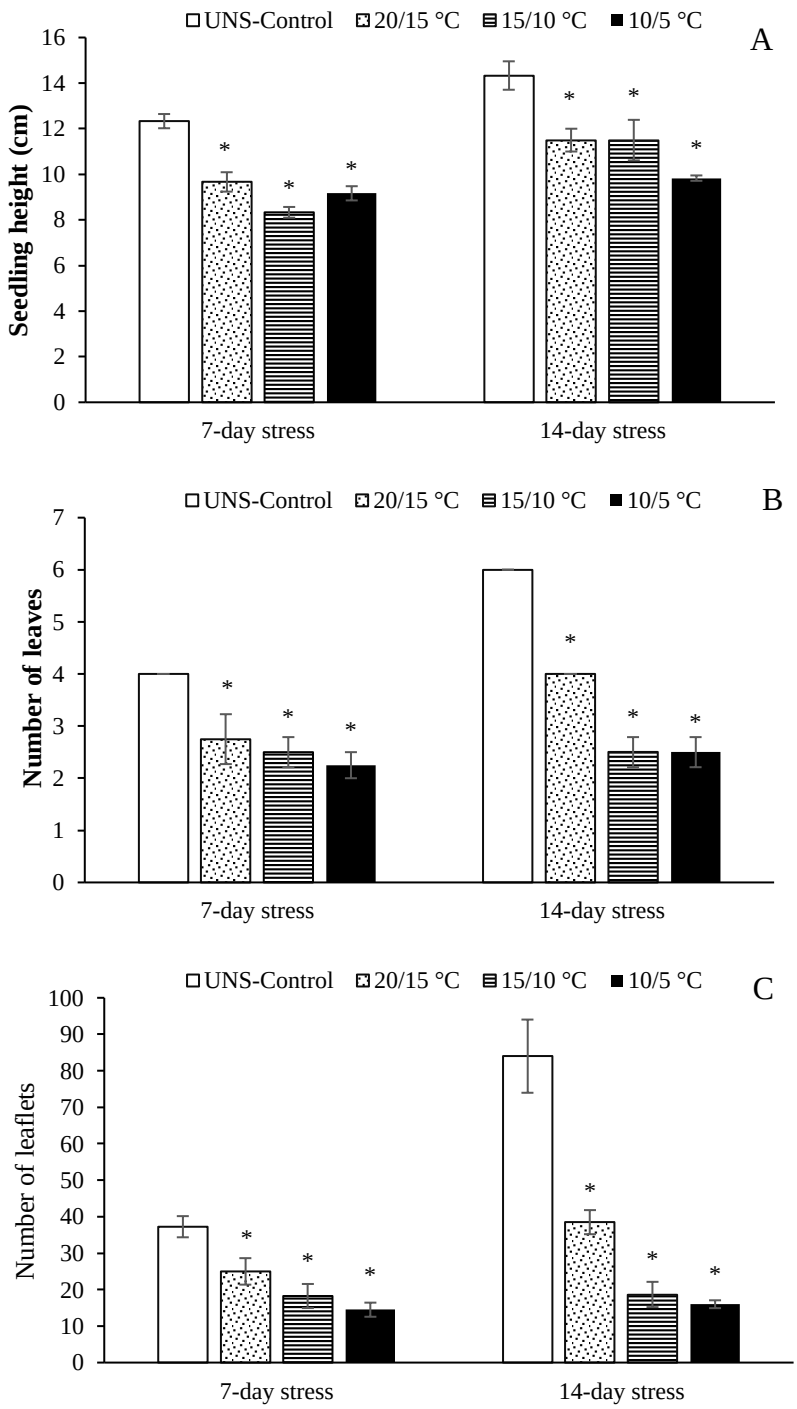

Figure 2. Seedling height (A), number of leaves (B), and number of leaflets (C) of moringa seedlings at 7-day stress (7DS) and 14-day stress (14DS) under unstressed condition (UNS-control), and growth chamber at 20/15 °C, 15/10 °C, and 10/5 °C. The error bar indicates ± standard error. * Indicates the difference from UNS-control through the Dunnett test ($p<0.05$).

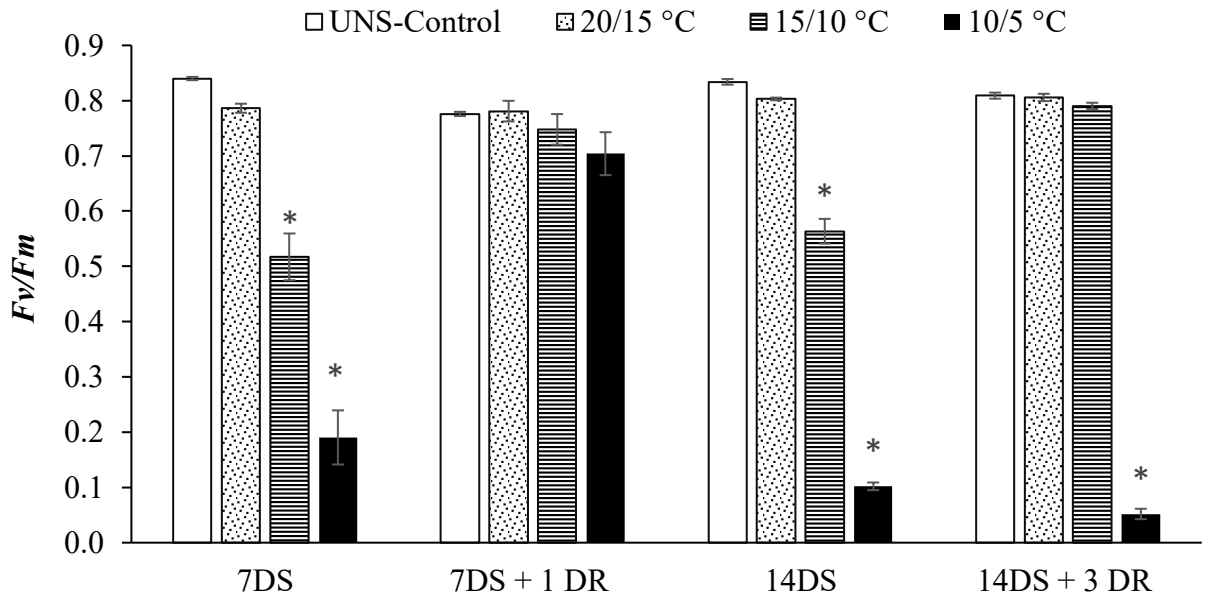

Figure 3. The maximum quantum yield efficiency of PSII *(Fv/Fm)* in moringa seedlings at UNS-control, 20/15 °C, 15/10 °C, and 10/5 °C after 7-day stress (7DS), 7-day stress + 1-day recovery (7DS + 1DR), 14-day stress (14DS), and 14-day stress + 3-day recovery (14DS + 3DR). The error bar indicates ± standard error. *Differs from the UNS-control through the Dunnett test ($p<0.05$).

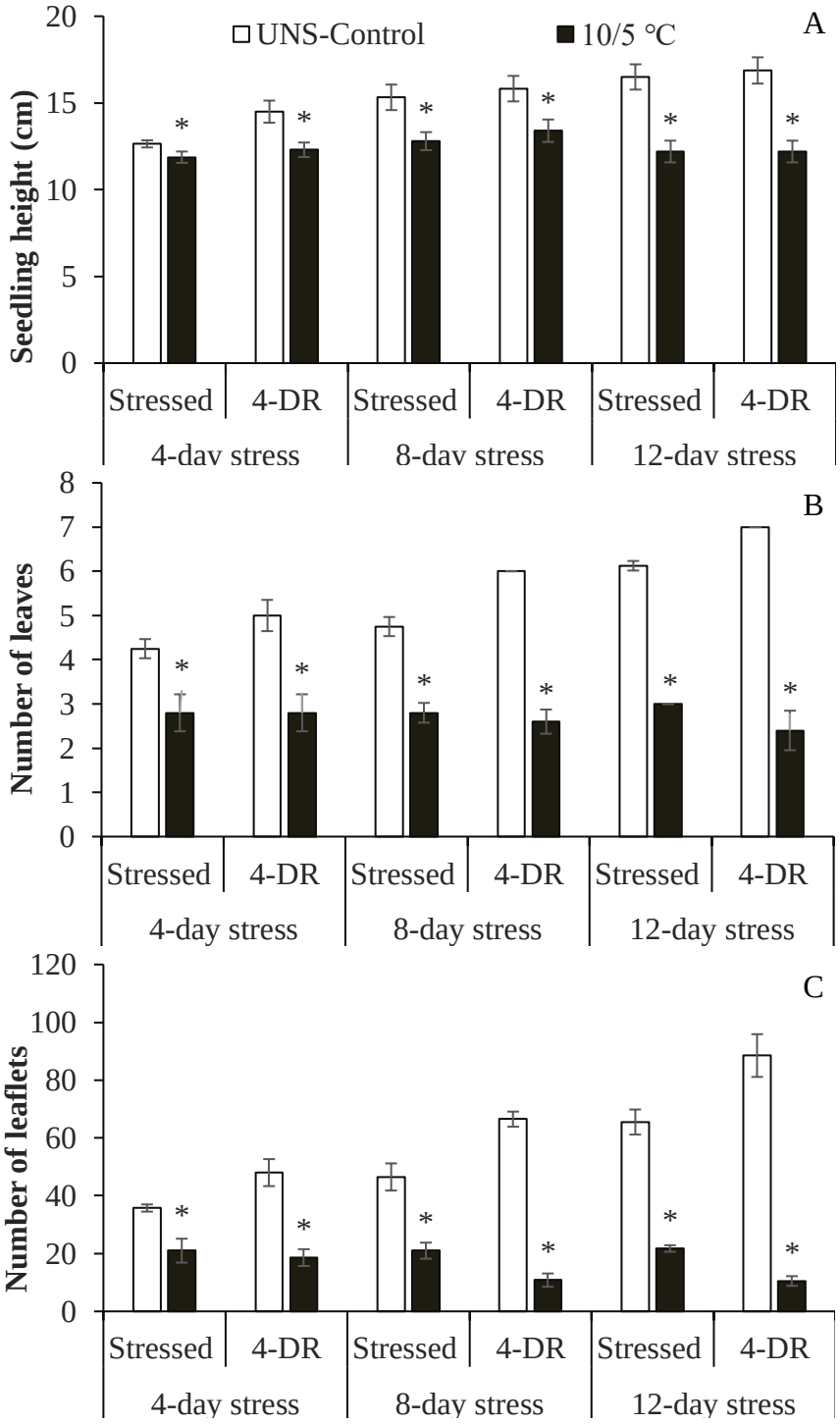

Figure 4. Seedling height (A), number of leaves (B), and number of leaflets (C) of moringa seedlings at 4-day stress, 8-day stress, and 12-day stress in a growth chamber at 10/5 °C and after 4-day recovery (4-DR) under unstressed condition (UNS-control). The error bar indicates ± standard error. *Differs from the GH-control by Student`s t-test ($p<0.05$).

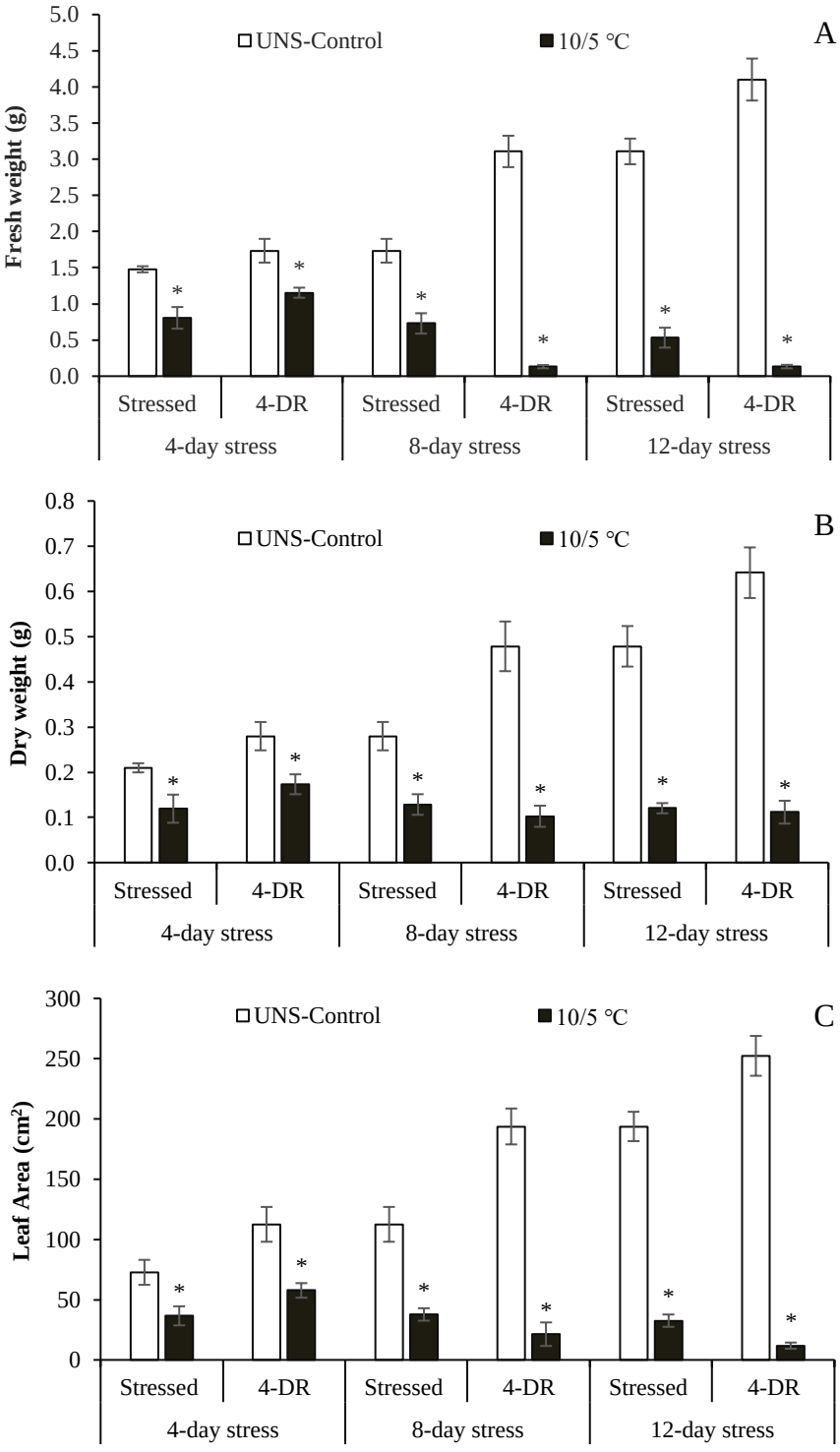

Figure 5. Fresh weight (A), dry weight (B), and leaf area (C) of moringa seedlings at 4-day stress, 8-day stress, and 12-day stress in a growth chamber at 10/5 °C and after 4-day recovery (4-DR) under unstressed condition (UNS-control). The error bar indicates ± standard error. *Differs from the GH-control by Student`s t-test ($p<0.05$).

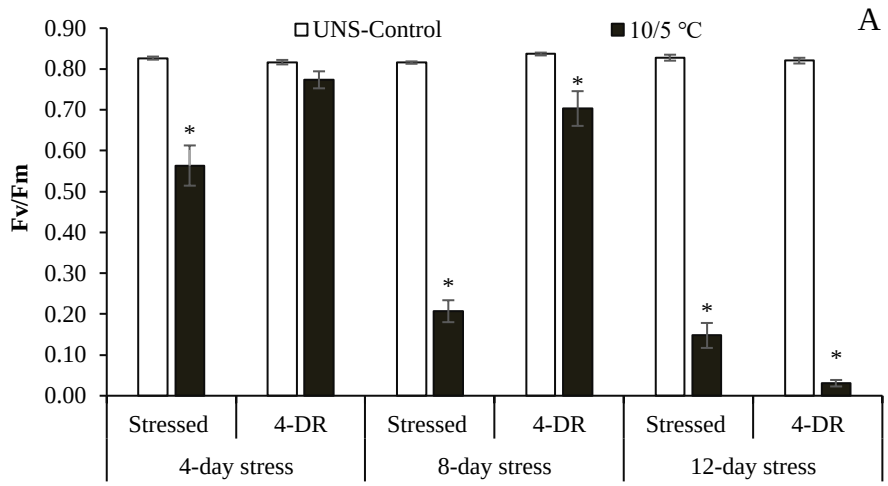

Figure 6. F_v/F_m(A) of moringa seedlings at 4-day stress, 8-day stress, and 12-day stress in a growth chamber at 10/5 °C and after 4-day recovery (4-DR) under unstressed condition (UNS-control). The error bar indicates ± standard error. *Differs from the GH-control by Student`s t-test ($p<0.05$).

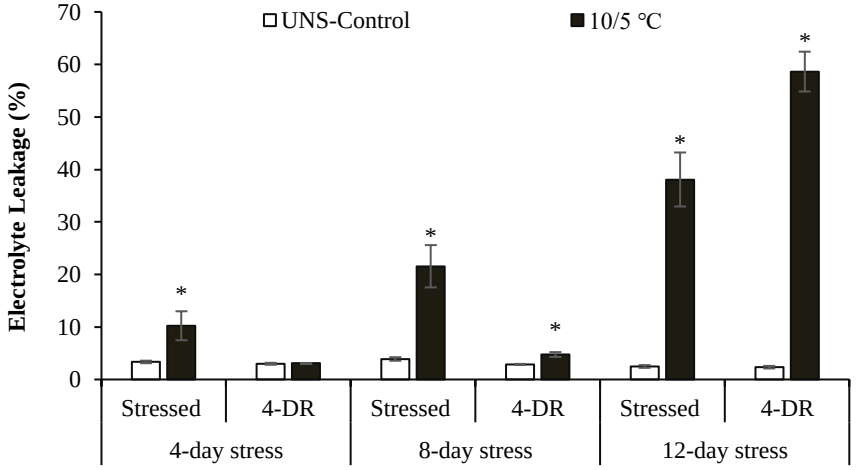

Figure 7: Electrolyte leakage (%) of moringa seedlings at 4-day stress, 8-day stress, and 12-day stress in a growth chamber at 10/5 °C and after 4-day recovery (4-DR) under unstressed condition (UNS-control). The error bar indicates ± standard error. *Differs from the GH-control by Student`s t-test ($p<0.05$).

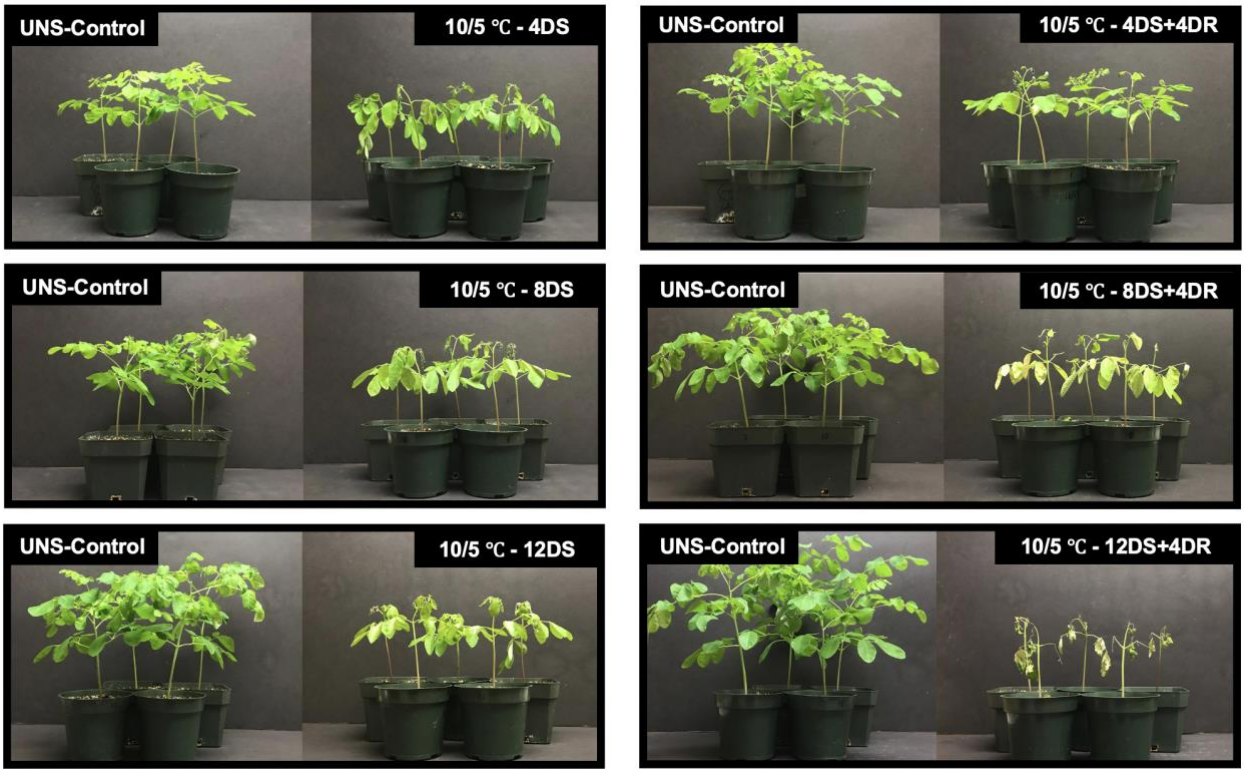

Figure 8: Visual symptoms of chilling response in *Moringa oleifera* Lam. seedlings under 4-day stress, 8-day stress, and 12-day stress at 10/5 °C and after 4-day recovery (4-DR) under unstressed condition (UNS-control).

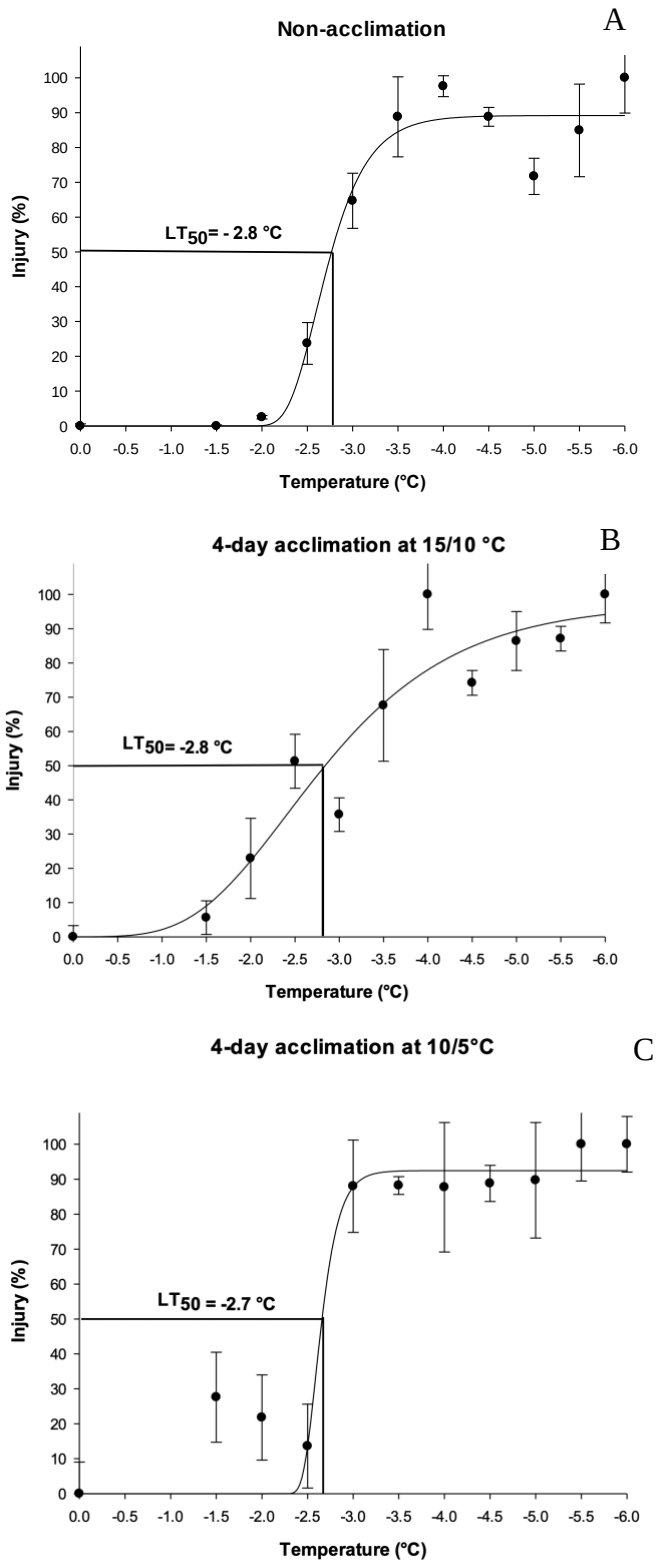

Figure 9: Freezing tolerance (FT_{50}) of non-acclimated (A), at 4-day acclimation at 15/10 °C (B), at 4-day acclimation at 10/5 °C (C) *Moringa oleifera* Lam seedlings.

7. MANUSCRIPT 4
GENETIC VARIABILITY OF MORINGA GENEBANK IN BRAZIL

Artigo submetido no periódico Genetic Resources and Crop Evolution

ABSTRACT
Genetic variability of Moringa Genebank in Brazil

Moringa oleifera Lam. is a tropical tree that belongs to Moringaceae family, and it is popularly known worldwide for its multiple uses. This study aimed to evaluate the genetic variability between the individuals from the Moringa Genebank of Embrapa Coastal Tablelands, Sergipe, Brazil. The Moringa Genebank is composed of 25 accessions represented by 177 genotypes, which 18 accessions were from an exchanged germplasm of the University of Florida, USA, and the others were from different states of Brazil. Leaves of each genotype were collected for DNA extraction and PCR analysis using 20 ISSR primers. A total of 144 bands were amplified and 100% of them were polymorphic. The average of expected heterozygosity (He) and Shannon's Index was 0.11 and 0.12, respectively. The highest divergence genetic was found between M4 and M18 accessions, and the closest pair of accession was M23 and M24, both from Brazil. The cluster analysis obtained through the Structure software divided moringa genotypes into two groups. Taken together, these results suggest low genetic diversity is found between the accessions of the Moringa Genebank. Therefore, genetic improvements need to be encouraged to ensure genetic conservation and plant development for this species.

Keywords: *Moringa oleifera* Lam.; genetic resources; ISSR markers.

7.1. Introduction

Moringa oleifera Lam., popularly known as drumstick tree, horseradish tree, miracle tree, and benzoil tree, belongs to the Moringaceae family, composed of 13 species, in which *Moringa oleifera* is the most popular one (Farooq and Koul 2020). Native to India and spread over tropical areas, *M. oleifera* grows well in Africa, China, and Mexico. *M. oleifera* is a fast-growing tree, which can reach up to12 m in height and be propagated through sexual and asexual. This plant demands low soil nutrients and water and is considered resistant to drought and diseases (Foidl et al. 2001). *M. oleifera* is composed of a slender stem with drooping branches, white flowers, tripinnate leaves, and white-brown seeds with papery wins. It is a deciduous perennial tree, recognized for its multipurpose since every part of this plant has a specific use (Pandey et al. 2011).

The leaves of this plant have a variety of nutrients, such as minerals, proteins, vitamins A, B1, B2, B3, C, and E, carotenoids, and flavonoids, among other bioactive compounds. Moreover, *M. oleifera* has been recently recognized as a superfood, being a valuable product for human and animal nutrition (Boopathi et al. 2021; Leone et al. 2015). This species has also been reported to have potential pharmacological effects, including antioxidants, anti-inflammatory, and anti-diabetic activity (Singh et al. 2020). *M. oleifera* seeds are used for water purification, in which the powder extracted from the seeds has natural coagulant properties for water treatment (Milla et al. 2021). Besides that, the seeds have a high content of oil, called "Ben", and can be used as a lubricant, in the culinary and cosmetic industries (Nair *et al.* 2021). *M. oleifera* crop is mostly cultivated by small farmers as a vegetable for domestic consumption (Pandey et al. 2011). In Brazil, *M. oleifera* was introduced as an ornamental tree circa 1950 and is now well distributed throughout the country, mainly in the northeast region (Rivas et al. 2013).

In view of the relevance of this species, it is important to ensure that genetic variability is preserved to improve the resources for future breeding programs. Moreover, collections and active germplasm banks are considered the key for this purpose (Leone et al. 2015). To conserve the genetic variability of *M. oleifera*, the Brazilian Agriculture Research Corporation (Embrapa) established the Moringa Genebank in 2009, located in Nossa Senhora das Dores,

SE, Brazil. Since then, new accessions have been implemented in the Moringa Genebank, which is currently composed of 25 accessions represented by 177 genotypes.

The evaluation of a germplasm collection represents an important step to promote conservation actions and select crucial traits in a crop improvement program (Nair et al. 2021). One way to assess genetic diversity is by evaluating phenotypic characteristics. However, there are some disadvantages related to the environmental influence. On the other hand, techniques based on DNA have been reported to be a relevant tool to identify polymorphisms and quantify the variability in a group of genotypes (Saini et al. 2013; Chaves-Bedoya et al. 2017).

Molecular markers based on Polymerase Chain Reaction (PCR) provide an analytical and reliable analysis of a plant's DNA. Among them, the Inter Simple Sequence Repeat (ISSR) primers are based on a microsatellite sequence, represents a fast and easy-to-handle technique with a high degree of polymorphism, and uses a low amount of DNA per reaction. In addition, this marker is multilocus and does not require prior knowledge of the DNA sequence (Henry 2013; Marwal, Sahu and Gaur 2013; REDDY et al. 2002). Therefore, this study aimed to evaluate the genetic diversity of moringa genotypes from a Moringa Genebank of the Brazilian Agriculture Research Corporation (Embrapa), using ISSR markers.

7.2. Material e Methods

7.2.1. Plant material

Moringa Genebank is established in the experimental field in the Nossa Senhora das Dores city, SE, Brazil (-10.463318, -37.191661). A total of 177 genotypes from 25 accessions, of which 18 are from exchanged germplasm of the University of Florida, USA, and the others are from different states of Brazil, such as Sergipe, Pernambuco, Paraíba, Rio Grande do Norte, and Ceará (Table 1). Leaves of each genotype were collected, stored in ice to be transferred to the laboratory, and kept at -80 °C until performing DNA extraction.

7.2.2. DNA extraction and purification

The DNA extraction was conducted based on Doyle and Doyle's (1990) methodology. The leaves (300 mg) were macerated using 1 mL of CTAB buffer. The DNA quantification was performed using a Nanodrop 2000c spectrophotometer (Thermo Scientific, USA). Samples were diluted in Tris EDTA at a concentration of 10 $ng.\mu L^{-1}$, and then kept at -20 °C to be used in PCR reactions.

7.2.3. ISSR-PCR amplification

The PCR reaction (20 μL for each sample) was composed of 1 μL of genomic DNA solution, 2 μL 10X PCR Buffer, 0.4 μL dNTP (10 mM), 0.6 μL $MgCl_2$ (50 mM), 1 μL of each primer, 0.2 μL Taq DNA polymerase Ludwig and 14.8 μL of ultrapure water.

The samples were placed in an Axygen Maxygene thermocycler that was programmed to amplify the PCR reaction. Each reaction consisted of a denaturation stage at 95 °C for 4 min, 45 cycles of amplification (denaturation at 94 °C for 45s, annealing at each primer temperature for 45s, and first extension at 72 °C for 2 min), followed by a final extension at 72 °C for 7 min. Twenty ISSR primers were tested (Table 2).

Samples after being amplificated by PCR reaction were placed for electrophoresis analysis. For this, an agarose gel 2 % was dissolved in TBE 1X (TRIS 89mM, boric acid 89 mM, EDTA 2.5 mM, pH 8.3) and placed under constant voltage of 182V, 91 mA, and 17W for 115 min, followed by staining with ethidium bromide solution (0.5 $\mu L.\ mL^{-1}$ of water) for 30 min. Then, the agarose gels were submitted to visualization under ultraviolet light using Gel doc L pix photo documentation equipment (Loccus Biotecnologia, Cotia, SP).

7.2.4. Data analysis

For each gel, the presence (1) and absence (0) of bands were transformed into a binary matrix. The genetic variability for each accession was assessed through the Shannon index (I) and expected heterozygosity (He) using Genalex software (Peakall and Smouse 2012).

The genetic variance between and within accessions was estimated using the Analysis of Molecular Variance (AMOVA) and the level of significace was determined with 9.999 permutations. The genetic difference between acessions (GST) was estimated (Nei 1973), which corresponds to the total of genetic variation, and its significance tested by 10.000 bootstraps. Additionally, the genetic distance of Nei between the accessions was estimated. The analyzes were carried out by the Genalex 6.5 software (Peakall and Smouse 2012).

The genetic distance between the accesses was evaluated by the genetic distance of Rogers (1972) and visualized by the construction of a dendrogram using the UPGMA (Unweighted Pair Group Method with Arithmetic Means) algorithm. The analysis was carried out using the poppr package (Kamvar et al. 2014) of the software R Core Team (2022). The FigTree 1.4.1 software was used to format the dendrogram. The principal coordinate analysis (PCoA), at the individual level, was carried out by the Genalex 6.5 software (Peakall and Smouse 2012).

The genetic structure of twenty accessions was estimated by the Bayesian analysis using the software Structure v.2.3.4 (Pritchard et al. 2000). The values of genetic groupings (k) varying from 1 to 25 (number of accesses) were tested and, 10 independent repetitions were performed for each k. Each replicate consisted of a burn-in period of 50,000 iterations, followed by 100,000 MCMC (Markov Chain Monte Carlo) iterations, assuming the mixture ancestry model and uncorrelated allele frequencies. The number of genetic groups (k) was identified by the ΔK method described by Evanno et al. (2005), implemented in Structure Harvester software (Earl and vonHoldt 2012). Accessions that showed membership values lower than 0.8 were considered mixed ancestry.

7.3. Results

The 20 ISSR primers used in our study generated 144 bands, which 100 % showed to be polymorphic (Table 2). The maximum and the minimum number of amplified bands ranged from 3 (UBC 818) to 10 (UBC 825 and 848), which was an average of seven bands per primer. The expected heterozygosity (He) showed variation from 0.03 (ISSR2) to 0.19 (UBC 825), with an average value of 0.11. Similarly, Shannon Index (I) ranged between 0.04 (ISSR2) and 0.25 (825), with an average value of 0.12.

The molecular variance was analyzed by AMOVA (Table 3). The results revealed 56% variance within accessions and 44% variance among accessions. Moreover, Nei's genetic distance matrix obtained with pairwise accessions is shown in Table 4. The highest genetic distance (0.310) was found between M4 and M18, both accession from the Florida exchange. On the other hand, the closest pair of accession is M23 and M24 (0.048), both from Brazil.

The relationship of all 177 genotypes was revealed by genetic distance of Rogers (Figure 1). Moringa genotypes were categorized into two groups. One cluster was composed of M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11, M12, and M13, in which genotypes M4.1, M4.2, M4.6, and M4.7 highlighted for being in a different subgroup of the others. The second cluster is composed of M14, M15, M16, M17, M18, M19, M20, M21, M22, M23, M24, and M25. Inside of that, three sub-clusters were identified, which include: most of the genotypes from M16 in one sub-cluster; M14 and M15 in the second sub-cluster; and the others in the third sub-cluster. In general, the cluster analysis did not correlate with the geographic origin of the accessions.

Similarly, the Bayesian analysis provided by the Structure software grouped moringa accesses in two broad clusters (K=2) (Figure 2). Group I (red) included all genotypes from the

Florida exchange (M1, M2, M3, M4, M5, M6, M7, M8, M9, M10, M11, M12, M13, whereas accession M11, M12, and M13 were categorized as admixture (values of membership higher than 0.2). On contrary, all accessions from Brazil (M19, M20, M21, M22, M23, M25) were ranked in Group II (green), except for M17 and M18 which were from the Florida exchange. The accessions M14, M15, and M16 were also categorized as an admixture for having membership values lower than 0.8.

Principal Coordinate Analysis (PCoA) with ISSR markers showed the distribution of genetic diversity among the accessions across the two axes. The percentage of variation explained was 26.01 % (axis1: 20.12% and axis2: 5.89%) (Figure 3) and agrees with both groups formed in the aforementioned cluster analysis.

7.4. Discussion

Previous studies have reported ISSR primers as an effective molecular marker to characterize genetic diversity in moringa (Hassan et al. 2020; Hassanein 2018; Rajalakshmi Rajalakshmi and Parida, 2019; Saini et al. 2013). In our study, all observed bands were polymorphic. Similar findings were reported by Hassanein (2018), in which 10 ISSR primers amplified 65 bands and 90.8 % were polymorphic for both species *Moringa oleifera* and *Moringa peregrina*. Saini et al. 2013 used six ISSR primers to detect the genetic variability among eight Indian cultivars of *Moringa oleifera,* which was considered the most adequate marker as compared to RAPD and cytochrome P_{450}-based markers, and the results showed a rate of 48.57 % of polymorphisms. Similarly, the genetic variability of 97 accessions of *Moringa oleifera* from India was assessed using 15 ISSR markers and amplified a total of 100 bands, showing 59.6% of polymorphism (Rajalakshmi et al. 2019).

The Shannon's Index infers from genetic diversity, in which more diversity is found in values close to 1 (Perry and McIntosh, 1991). In our study, the average of Shannon's Index was 0.16, which indicates a low genetic diversity since it closer to zero and this index ranges from 0 to 1 (Silva et al. 2015). Another indicator of genetic diversity is the expected heterozygosis (He) which varied from 0.03 to 0.15 (mean of 0.11), indicating low variability in the 177 genotypes of moringa. Natural populations normally have a He values higher than zero due to the incorporation of new alleles by crossing (Silva et al. 2014). Similarly, Chaves-Bedoya et al. (2017) also found low genetic diversity in 45 accessions from Colombia, in which values of He was ranging between 0.13 and 0.29. The authors reported that the genotypes from Colombia were probably from a single population or a few populations.

On contrary was observed when evaluating genetic materials from India where more genetic diversity is found since it is Moringa's origin center (Muluvi et al. 1999). A high level of genetic diversity was found at evaluating seven advanced breeding lines from different locations in India (Kumar et al. 2017). Ganesan et al. (2014) used 19 primers SSR to assess the genetic diversity among 300 genotypes of 12 populations from northern (Himachal Pradesh) and southern (Tamil Nadu) India. They also found a high genetic diversity in the Indian collection and reinforce the idea moringa was originated in north India and was progressively establish in southern part where it became more diverse. In another study using genotypes of natural populations from India, Malawi and Kenya showed that Nei's average values ranged from 0.040 (Kenya) and 0.122 (Indian population), and the highest level of genetic diversity was found within Indian populations (Muluvi et al. 1999). In our study, the highest coefficient of Nei's Genetic Distance was found between two accessions from the Florida exchange.

The AMOVA results indicated that the greatest genetic variability was found within accession (56 %) (Table 3). Similar was reported by Ganesan et al. (2014) and Rajalakshmi et al. (2019), in which the AMOVA's analysis showed 86% and 95% of the variation was found within the population, respectively. Since moringa is a cross-pollinated plant is expected that most variation is found within the population (Leone et al. 2015). Moreover, different factors

may affect the genetic variability in moringa populations, such as aleatory mating patterns, genetic drifts caused by changes in allelic frequency, spontaneous mutation, and migration events of alleles within the population (Lakshmidevamma et al. 2021).

The Bayesian analysis provided by Structure software is a well-established tool to obtain information about population structure using bands of molecular markers (Pritchard et al. 2000). In our study, the Bayesian analysis was also a valuable grouping method to categorize moringa genotypes, being possible to identify which genotypes are considered an admixture by stablishing a threshold score (Figure 2). Even though Moringa Genebank accessions were divided into two groups and most of the accession from the University of Florida were allocated in group 1, some of them (M11, M12, M13, M14, M15, M16, M17, and M18) share similarities with accessions collected in Brazil, which belongs to group 2. Similar observations were obtained from the PCoA plot that together with structure data and cluster dendrogram can provide a more reliable outlook from the results (Figure 3).

A possible explanation for the lack of correlation between genetic variability versus geographic origin can be explained by the origin center of Moringa. Most genetic studies with moringa are performed in Asia, more specifically in India since its origin center is the Northwestern of India (Muluvi et al. 1999; Saini et al. 2013; Ganesan et al. 2014; Kumar et al. 2017; Ravi et al. 2020). In addition, studies with cultivated and natural accessions to quantify the genetic diversity of moringa across the world are considered mere, although the conservation of genetic resources for this species by using germplasm banks is increasing (Boopathi et al. 2021).

Despite a wide cultivation of moringa in America, there are only a few studies that attempted to assess the genetic variation of genotypes from Mexico (Avila-Treviño et al. 2017) and Colombia (Chaves-bedoya et al. 2017). Previously, the first molecular characterization of the Moringa Genebank was conducted with only 16 accessions from exchanged germplasm of University of Florida, using RAPD markers (Silva et al. 2012). Since then, new accessions of moringa collected in Brazil were introduced and our study represents the first molecular evaluation of genotypes from Brazil using ISSR primers for this specie.

In the literature, it is reported that moringa was introduced as an ornamental tree in United States in 1915, more specifically in southern Florida, with seeds from Cuba and Nicaragua. The Director of ECHO (Educational Concerns of Hunger Organization) became interested in moringa due to its adaptability and distributed seeds to other countries such as Haiti and Brazil. In Brazil, the author reported a seed shipment to the state of Maranhão, which resulted in 25,000 trees planted (Morton 1991).

Shahzad et al. (2013) evaluated the genetic diversity in 131 accessions from a wild population in Pakistan and 30 accessions obtained from ECHO (Florida), which were from nine different countries: Haiti, Mexico, Belize, USA (Florida), Zimbabwe, Mozambique, Tanzania, Senegal, and India. Interestingly, even though there was a great genetic diversity in the wild collection from Pakistan, low genetic diversity was found in the accessions obtained from ECHO. Consequently, the authors suggested that those accession are probably from the same population or from a few populations since moringa was introduced from India to different countries by traders or immigrants and ECHO received seeds from theses farmers. It is reported that the fact of introduced populations may show a low genetic diversity can be explained by using related accessions when they were introduced into a new place. In addition, high selection factors can be operating to reduce even more the genetic diversity in those populations (Muluvi et al. 1999). Our findings also agree with this same pattern of multiple ancestral populations of moringa with a narrow geographic origin may have been spread over different countries.

7.5. Conclusions

Taken together, the ISSR markers were effective to assess the genetic variability in *Moringa oleifera* Lam. Overall, the 25 accessions of Moringa Genebank were distributed into two large groups, however, it was not possible grouping them geographically. In addition, low genetic diversity was found between the accessions. The information obtained from this study can be used in management actions of the Moringa Genebank to improve genetic resources aiming not only to ensure conversation strategies for this species but also future breeding programs.

7.6. References

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Tables and Figures

Table 1. Moringa accessions (*Moringa oleifera* Lam.) from the Active Germplasm Bank of Embrapa Coastal Tablelands

Location	Accession	Number of genotypes
University of Florida, United States	M1	8
	M2	8
	M3	8
	M4	8
	M5	8
	M6	10
	M7	8
	M8	5
	M9	7
	M10	9
	M11	6
	M12	7
	M13	4
	M14	7
	M15	5
	M16	9
	M17	9
	M18	2
Fortaleza, Ceará, Brazil	M19	2
Mossoró, Rio Grande do Norte, Brazil	M20	3
Petrolina, Pernambuco, Brazil	M21	7
Aracaju, Sergipe, Brazil	M22	7
Frei Paulo, Sergipe, Brazil	M23	10
Itabaiana, Sergipe, Brazil	M24	10
Queimadas, Paraíba, Brazil	M25	10
Total		177

Table 2. ISSR primers, anneling temperature, and the number of polymorphic bands of 25 accessions from the Moringa Germoplasm Bank of Embrapa Coastal Tablelands. He: Expected Heterozygosity; I: Shannon's Index.

Primer	Sequence	Annealing (°C)	Total of bands	Polymorphic bands	Polymorphism (%)	He	I
UBC 807	5' AGA GAG AGA GAG AGA GT 3'	47.0	5	5	100	0.09	0.13
UBC 809	5' AGA GAG AGA GAG AGA GG 3'	57.2	4	4	100	0.15	0.21
UBC 813	5' CTC TCT CTC TCT CTC TT 3'	44.6	4	4	100	0.12	0.18
UBC 811	5' GAG AGA GAG AGA GAG AC 3'	46.8	6	6	100	0.07	0.10
UBC 816	5' CAC ACA CAC ACA CAC AT 3'	54.8	8	8	100	0.13	0.19
UBC 818	5' CAC ACA CAC ACA CAC AG 3'	57.2	3	3	100	0.10	0.15
UBC 823	5' TCT CTC TCT CTC TCT CC 3'	57.2	8	8	100	0.11	0.15
UBC 825	5' ACA CAC ACA CAC ACA CT 3'	54.8	10	10	100	0.17	0.25
UBC 826	5' ACA CAC ACA CAC ACA CC 3'	57.2	9	9	100	0.10	0.15
UBC 827	5' ACA CAC ACA CAC ACA CG 3'	57.2	9	9	100	0.07	0.11
UBC 845	5' CTC TCT CTC TCT CTC TRG 3'	58.8	8	8	100	0.09	0.13
UBC 848	5' CAC ACA CAC ACA CAC ARG 3'	58.8	10	10	100	0.15	0.23
UBC 855	5' ACA CAC ACA CAC ACA CYT 3'	53.1	9	9	100	0.11	0.18
UBC 856	5' ACA CAC ACA CAC ACA CYA 3'	56.5	7	7	100	0.14	0.21
UBC 860	5' TGT GTG TGT GTG TGT GRA 3'	46.9	9	9	100	0.15	0.22
UBC 864	5' ATG ATG ATG ATG ATG ATG 3'	50.8	7	7	100	0.05	0.08
ISSR 1	CAC ACA CAC ACA GG	52.6	8	8	100	0.15	0.23
ISSR 2	CTC TCT CTC TCT CTC TAC	57.6	4	4	100	0.03	0.04
ISSR 4	CAC ACA CAC ACA AC	49.7	6	6	100	0.11	0.16
ISSR 6	CAC ACA CAC ACA AG	49.7	7	7	100	0.12	0.18
			7	7	100	0.11	0.16

Table 3. Analysis of molecular variance (AMOVA) showing the genetic variation within and among accessions from Moringa Germplasm Bank of Embrapa Coastal Tablelands based on 20 ISSR primers. Df = degree of freedom, SS= sum of squares, MS =mean of squares, Est. var. = estimate of variance, % = percentage of total variation.

Source of variation	df	SS	MS	Est. Var.	%	GST
Among Accessions	24	1597.69	66.57	7.99	44 %	0.440 ***
Within Accessions	152	1549.39	10.19	10.19	56 %	
Total	176	3146.98	-	18.92	100 %	

***p<0.001

Table 4. Matrix using the coefficient of Nei's Genetic Distance from the 25 accessions of Moringa Germoplasm Bank of Embrapa Coastal Tablelands.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
0.000																									1
0.102	0.000																								2
0.085	0.081	0.000																							3
0.102	0.124	0.066	0.000																						4
0.117	0.116	0.099	0.071	0.000																					5
0.111	0.103	0.144	0.142	0.134	0.000																				6
0.097	0.108	0.121	0.103	0.089	0.052	0.000																			7
0.115	0.089	0.098	0.107	0.093	0.097	0.068	0.000																		8
0.131	0.105	0.140	0.129	0.091	0.099	0.080	0.088	0.000																	9
0.117	0.103	0.108	0.111	0.098	0.097	0.076	0.095	0.053	0.000																10
0.142	0.145	0.133	0.125	0.111	0.158	0.139	0.125	0.130	0.114	0.000															11
0.144	0.179	0.145	0.149	0.173	0.178	0.159	0.133	0.167	0.125	0.103	0.000														12
0.130	0.157	0.117	0.129	0.154	0.180	0.157	0.151	0.148	0.116	0.081	0.083	0.000													13
0.209	0.212	0.192	0.211	0.225	0.218	0.234	0.251	0.222	0.201	0.169	0.191	0.131	0.000												14
0.223	0.240	0.235	0.258	0.243	0.236	0.269	0.294	0.242	0.213	0.191	0.203	0.166	0.089	0.000											15
0.192	0.182	0.204	0.211	0.232	0.183	0.225	0.237	0.224	0.202	0.186	0.194	0.174	0.131	0.118	0.000										16
0.235	0.235	0.261	0.266	0.254	0.237	0.249	0.255	0.225	0.220	0.192	0.218	0.178	0.131	0.150	0.081	0.000									17
0.246	0.264	0.267	**0.310**	0.262	0.265	0.269	0.285	0.277	0.268	0.241	0.265	0.205	0.161	0.164	0.171	0.097	0.000								18
0.264	0.227	0.240	0.263	0.232	0.259	0.267	0.271	0.259	0.241	0.194	0.232	0.188	0.141	0.175	0.157	0.128	0.100	0.000							19
0.206	0.192	0.199	0.240	0.237	0.209	0.224	0.260	0.224	0.207	0.197	0.250	0.226	0.178	0.171	0.163	0.132	0.136	0.120	0.000						20
0.228	0.231	0.222	0.240	0.279	0.265	0.254	0.293	0.241	0.228	0.207	0.232	0.137	0.126	0.143	0.129	0.102	0.159	0.178	0.153	0.000					21
0.219	0.211	0.251	0.264	0.257	0.232	0.258	0.264	0.237	0.221	0.208	0.239	0.176	0.114	0.116	0.117	0.087	0.088	0.111	0.130	0.093	0.000				22
0.196	0.189	0.207	0.233	0.251	0.222	0.206	0.228	0.213	0.209	0.167	0.218	0.156	0.127	0.147	0.134	0.110	0.128	0.134	0.106	0.076	0.070	0.000			23
0.218	0.206	0.207	0.228	0.232	0.236	0.222	0.246	0.214	0.210	0.163	0.206	0.164	0.127	0.146	0.114	0.105	0.134	0.116	0.122	0.077	0.077	**0.048**	0.000		24
0.238	0.225	0.225	0.217	0.254	0.250	0.228	0.268	0.221	0.211	0.189	0.223	0.167	0.128	0.175	0.156	0.152	0.179	0.155	0.161	0.073	0.089	0.059	0.050	0.000	25

Figure 1. Matrix using the coefficient of ROGERS from the 25 accessions of Moringa Germoplasm Bank of Embrapa Coastal Tablelands.

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Figure 2. The representation of 25 accessions from Moringa Genebank of Embrapa Coastal Tablelands divided into two groups (K=2) by the Structure software using 20 ISSR markers. Group 1 = read and Group 2 = green.

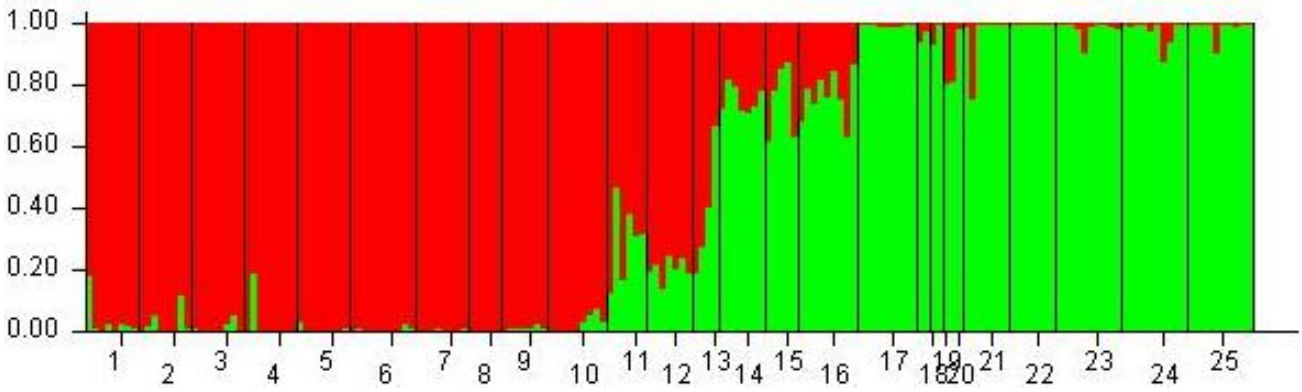
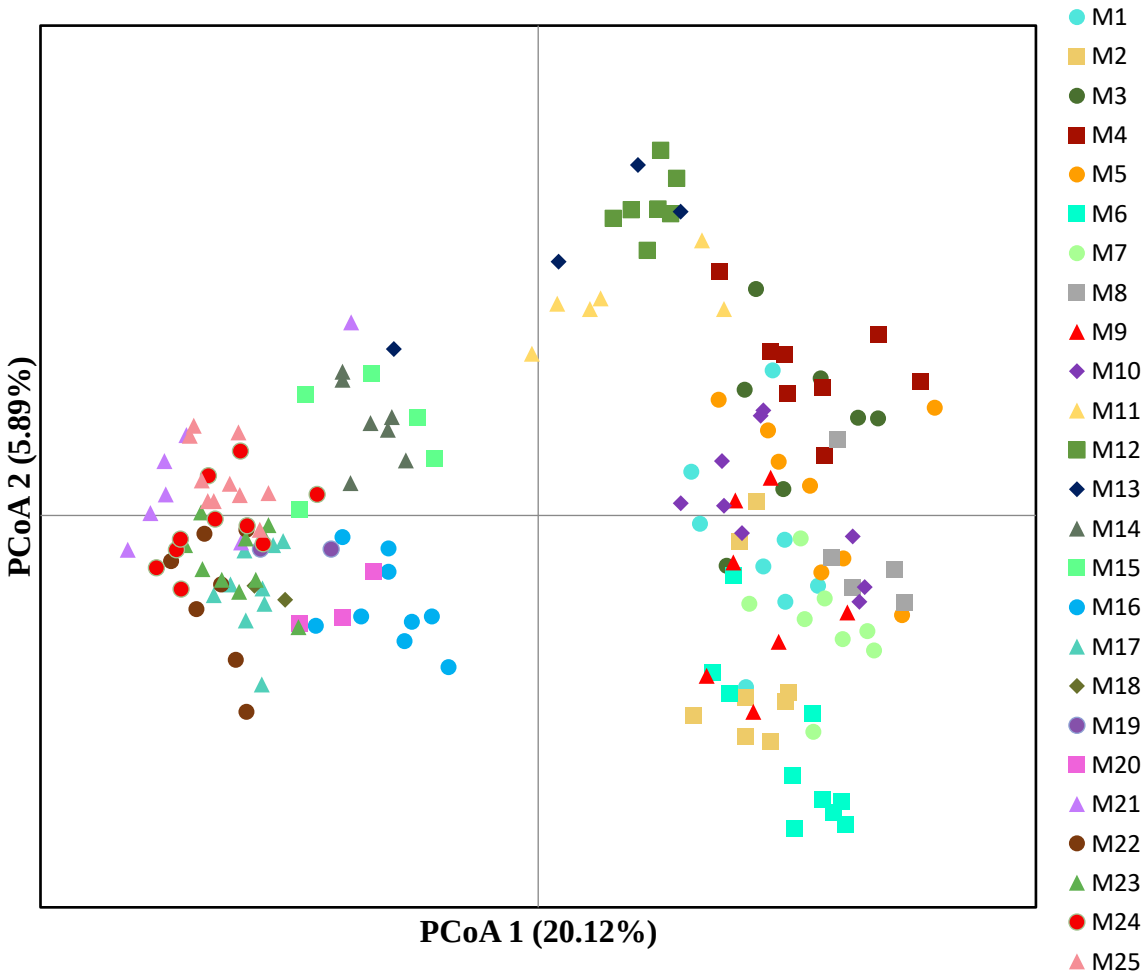


Figure 3. Principal coordinate analysis (PCoA) in 25 accessions from Moringa Germoplasm Bank of Embrapa Coastal Tablelands.



8. CONSIDERAÇÕES FINAIS

Tendo em vista as diversas potencialidades da *Moringa oleifera* Lam. é relevante destacar a importância dos estudos voltados para a difusão do uso do seu extrato foliar como bioestimulante natural, podendo ser aplicado no tratamento de sementes como um produto eficiente, sustentável e de baixo custo para promover o crescimento de plantas.

Além disso, destaca-se a importância do entendimento da ecofisiologia dessa espécie e de como a moringa tolera ao estresse de baixas temperaturas, fator este considerado um dos mais limitantes para a sua expansão geográfica. Nesse sentido, a resposta da espécie quanto ao estresse de frio é regulada pela intensidade e duração do estresse, o regime de temperatura 10/5 ℃ causou redução significativa do seu crescimento e da sua capacidade fotossintética e o período de exposição por 8 dias provocou uma lesão irrecuperável as suas membranas celulares e seu aparato fotossintético. A moringa demonstrou ainda tolerar a formação de gelo em seus tecidos, com o LT50 de -2,8 ℃. No entanto, não apresentou habilidade para aclimatação ao frio.

Por fim, os 20 marcadores moleculares ISSR se mostraram eficientes para avaliar a variabilidade genética entre os acessos de moringa do BAG Moringa da Embrapa Tabuleiros Costeiros. Além disso, foi identificado nível de variabilidade genética baixa entre os 25 acessos avaliados, sendo de forma geral divididos em dois grandes grupos, no entanto, não foi possível separá-los geograficamente.