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UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

MENTHA X *PIPERITA*, *OCIMUM BASILICUM* E *SALVIA DESERTA*, (LAMIACEAE): ABORDAGENS FISIOLÓGICAS E FITOQUÍMICAS

JENNIFER BÚFALO

Tese apresentada ao Instituto de Biociências, Câmpus de Botucatu, UNESP, para obtenção do título de Doutor em Ciências Biológicas (Botânica) Área de concentração: Fisiologia Vegetal.

BOTUCATU – SP

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Resumo e Abstract

BUFALO, J. ***MENTHA* X *PIPERITA*, *OCIMUM BASILICUM* E *SALVIA DESERTA*, (LAMIACEAE). ABORDAGENS FISIOLÓGICAS E FITOQUÍMICAS.** 2015, 89 p. - INSTITUTO DE BIOCIÊNCIAS, UNESP – UNIVERSIDADE ESTADUAL PAULISTA, BOTUCATU.

RESUMO - As plantas medicinais e aromáticas como hortelã-pimenta (*Mentha* x *piperita*), manjericão doce (*Ocimum basilicum*) e salvia (*Salvia deserta*) possuem grande importância no contexto mundial devido à demanda das indústrias de alimentos, químicas e farmacêuticas. Hortelã-pimenta, além de ser utilizada pela medicina popular, é cultivada principalmente pela extração de óleo essencial. O estudo realizado objetivou avaliar se o estresse osmótico, aplicado em duas concentrações de polietilenoglicol (PEG) em curto período de tempo, influencia na anatomia e ultraestrutura foliar e interfere no padrão fisiológico da *M.* x *piperita,* modificando o perfil do óleo essencial. Os resultados indicaram que as respostas ao estresse osmótico, foram dose dependente, pois as plantas submetidas ao PEG 50 g L^{-1} mantiveram os aspectos estruturais e funções metabólicas semelhantes às plantas do tratamento controle. Plantas submetidas ao PEG 100 g L^{-1}, apresentaram alterações anatômicas, danos ultraestruturais, como degradação e lise de organelas, as quais estão de acordo com a redução do potencial água das folhas e das trocas gasosas, aumento no conteúdo de açúcares totais e ativação no sistema de defesa antioxidante. Essas plantas apresentaram diminuição do conteúdo e qualidade do óleo essencial. O manjericão doce, planta medicinal importante utilizada principalmente na culinária, foi cultivado em casa de vegetação em sistema de fertilização orgânica e convencional, em duas doses de nitrogênio (150 e 250 kg ha^{-1} N). Os resultados revelaram que plantas cultivadas com fertilizante convencional na dose de 250 kg ha^{-1} N apresentaram maior massa fresca. Os tratamentos aplicados não afetaram o conteúdo, produção e composição do óleo essencial, sendo o linalol, o composto majoritário encontrado no estudo. Os resultados demostraram que independente do fertilizante, orgânico ou convencional, não houve modificação da composição do óleo essencial. A bioprospecção realizada com raízes de *S. deserta* identificou a presença de quatro diterpenos com atividades biológicas. *Taxodione* apresentou atividade antileishmanicida, antifúngica e antimicrobiana e o *ferruginol* apresentou a maior atividade (24-h IC_{50} 1.29 mg L^{-1}) contra bactéria presente em peixes, *Streptococcus iniae*. A fração do extrato bruto qual continha os compostos isolados *7-O-acetylhorminone* e *horminone* mostraram forte atividade antibacteriana (1.28 µg mL^{-1} para *Staphylococcus aureus* e 1.12 µg mL^{-1} para *S. Aureus resistente á meticilina* (MRSA) do que os compostos testados isoladamente. *7-O-acetylhorminone* e *horminone* exibiram ação sinérgica contra MRSA (FIC 0.2 µg mL^{-1}) e *horminone* apresentou melhor atividade contra *S. aureus* em relação aos outros compostos isolados de raízes de *S. deserta*. O estudo mostra que raízes de *S. deserta* são fontes potenciais de diterpenos com atividades biológicas e possibilidade para o desenvolvimento de novos compostos dessa planta com atividade pesticida. De modo geral, o estudo permite concluir que o metabolismo das plantas medicinais e aromáticas responde à variação das condições abióticas, déficit hídrico e nutrição, modificando o óleo essencial sintetizado, cuja bioprospecção pode revelar atividades biológicas de grande importância econômica.

Palavras-chave: hortelã-pimenta, salvia, manjericão, óleo essencial, ultraestrutura, diterpenos.

BUFALO, J. ***MENTHA* X *PIPERITA*, *OCIMUM BASILICUM* AND *SALVIA DESERTA*, (LAMIACEAE). PHYTOCHEMICAL AND PHYSIOLOGICAL APPROACHES.** 2015, 89 p. - INSTITUTO DE BIOCIÊNCIAS, UNESP – UNIVERSIDADE ESTADUAL PAULISTA, BOTUCATU.

ABSTRACT - Medicinal and aromatic plants such as peppermint (*Mentha x piperita*), sweet basil (*Ocimum basilicum*) and salvia (*Salvia deserta*) have great importance in the global context due to the demand of the food, chemical, and pharmaceutical industries. Peppermint is used by folk medicine and is grown mainly for essential oil extraction. The study investigated whether osmotic stress induced by two polyethyleneglycol (PEG) levels, in a short time, in peppermint changes the physiological pattern, anatomy, leaf ultrastructure and essential oil content and composition. The results indicated that osmotic stress responses were dose dependent, as plants subjected to PEG 50 g L^{-1} maintained structural features and metabolic functions similar to those of control plants. Plants exposed to PEG 100 g L^{-1} showed anatomical changes and ultrastructural damage as degradation and organelles lysis, which are in agreement with the low leaf water potential, gas exchange reduction, increase of total sugars, and activity of antioxidant enzymes. These plants showed lower content and quality of essential oil. Sweet basil, an important medicinal plant used mainly in culinary arts, was grown in a greenhouse using both organic and conventional fertilization systems with two nitrogen rates each (150 and 250 kg N/ha). The results showed that the highest fresh weight was obtained from the plants grown with conventional fertilizer at a rate of 250 kg N/ha. The treatments did not affect the essential oil content, yield, and composition and linalool was the major compound found in the study. The results showed that regardless of fertilizer, organic or conventional, there was no change in the composition of the oil. The bioprospecting conducted with *S. deserta* roots identified the presence of four diterpenes with biological activities. Taxodione showed leishmanicidal, antifungal, and antimicrobial activity, and the ferruginol displayed the greatest activity (24-h IC_{50} 1.29 mg/L) against the fish pathogenic bacteria *Streptococcus iniae*. The crude extract fraction which contained the isolated compounds 7-*O*-acetylhorminone and horminone showed stronger in vitro antibacterial activity (1.28 µg/mL for *Staphylococcus aureus* and 1.12 µg/mL for methicillin-resistant *S. aureus* - MRSA) than the compounds tested alone. 7-*O*-Acetylhorminone and horminone exhibited a synergistic effect against MRSA (FIC 0.2 µg/mL), and horminone had better activity against *S. aureus* with respect to other compounds isolated from *S. deserta* roots. The study shows that *S. deserta* roots are a good source of diterpenoids with biological activities and that are possibilities for the development of novel antipesticidal compounds from this plant. Overall, the study suggests that the metabolism of medicinal and aromatic plants respond to changes in abiotic conditions, water deficit, and nutrition by modifying the essential oil synthesized, where bioprospecting can reveal biological activities of great economic importance.

Keywords: peppermint, salvia, basil, essential oil, ultrastructure, diterpenes.

Introdução e Revisão Bibliográfica

Introdução

As plantas produzem grande diversidade de compostos do metabolismo secundário, a partir da biossíntese provenientes de compostos do metabolismo primário. Esses compostos secundários, encontrados em concentrações relativamente baixas e em determinados grupos de plantas, podem apresentar marcantes atividades biológicas (SANTOS, 2007). A biossíntese desses compostos depende de inúmeros fatores do ambiente, como condições climáticas, sazonais, geográficas e de solo (GOBBO-NETO; LOPES, 2007). No entanto, os fatores genéticos também interferem na produção de compostos do metabolismo secundário (TRAPP; CROTEAU, 2001). Os metabolitos secundários estão envolvidos na resistência a pragas e doenças, na atração de polinizadores, na interação com microorganismos, entre outros (SANTOS, 2007).

Atualmente, o interesse nos metabólitos secundários tem aumentado devido à grande importância que desempenham na indústria farmacêutica, alimentícia, bebidas, perfumaria e cosméticos (SANTOS, 2007). Entretanto, ainda são necessários estudos que integrem conhecimento sobre fisiologia, anatomia e morfologia das plantas, bem como química de produtos naturais, como forma de identificar os compostos naturais, compreender os fatores que interferem no crescimento e desenvolvimento de plantas que produzem princípios ativos com interesse medicinal. Espera-se que as variáveis fisiológicas, anatômicas e químicas avaliadas neste trabalho, contribuam para melhor entendimento do metabolismo das espécies da família Lamiaceae, *Mentha* x *piperita, Ocimum basilicum* e *Salvia deserta* produtoras de metabólitos secundários. Assim, como em outras famílias de espécies medicinais, existe crescente busca por espécies pouco exploradas e produtoras de metabólitos secundários, cuja bioprospecção revele compostos com atividades biológicas ou ainda estudos que abordem o cultivo de plantas medicinais visando melhorar a produção e qualidade de metabólitos de óleos essenciais.

Um estudo sobre o estresse osmótico induzido pelo PEG em *Mentha* x *piperita* L.: aspectos estruturais e respostas metabólicas é apresentado no capítulo I.

O capítulo II refere-se a efeitos da fertilização orgânica *versus* convencional em manjericão doce (*Ocimum basilicum* L.) cultivado em casa de vegetação.

No capítulo III o estudo realizado aborda atividades antimicrobianas e antileishimania de diterpenóides isolados de raízes de *Salvia deserta* Shang.

Revisão Bibliográfica

Família Lamiaceae

A família Lamiaceae ou Labiatae pertencente a ordem Lamiales, que também inclui outras famílias como Verbenaceae, Scrophulariaceae e Acanthaceae (TUCKER; NACZI, 2006), é conhecida como família da menta (JUDD et al., 2009) e representada por 252 gêneros, com 6.800 a 7.200 espécies (JUDD et al,. 2009; RAJA, 2012). Recentes estudos sobre morfologia química e filogenia molecular têm identificado alterações na classificação da família, resultando na adição de gêneros anteriormente incluídos na família Verbenaceae (JUDD et al., 2009; HARLEY et al., 2012).

Essa família possui distribuição cosmopolita (JUDD et al., 2009), suas espécies são nativas principalmente do Mediterrâneo, embora algumas tenham origem na Austrália, Sudoeste da Ásia e América do Sul. Atualmente ocorrem em regiões tropicais e temperadas, em todo o mundo, exceto na Antártida (HARLEY et al., 2012).

As espécies podem ser ervas, arbustos ou árvores e apresentam importância econômica por conterem óleos essenciais ou também pela utilização como especiarias, incluindo gêneros como *Mentha, Lavandula, Rosmarinus*, *Salvia, Ocimum, Tymus* e *Origanum* (JUDD et al., 2009). As principais espécies estão alocadas na subfamília Nepetoideae que representa 47% das espécies (TUCKER; NACZI, 2006). Entre elas, *Salvia officinalis* L., *Rosmarinus officinalis* L., *Mentha* L. spp., *Thymus vulgaris* L., *Origanum* L. spp., *Satureja hortensis* L., *Monarda* L. spp., *Melissa officinalis* L., *Lavandula* L. spp., *Aeollanthus suaveolens* Mart. ex Spreng., *Ocimum* spp. e *Plectranthus* L'Hér. spp. e são conhecidas por produzirem óleos aromáticos com grande importância comercial ou medicinal (HARLEY et al., 2012).

Atualmente o conhecimento dos inúmeros compostos químicos provenientes de espécies da família Lamiaceae é muito extenso, sendo dominado por óleos essenciais, especialmente por monoterpenos e sesquiterpenos encontrados em gêneros de importância econômica (WU et al., 2012). No entanto, constituintes químicos, como diterpenos, triterpenos, fenólicos, e outros compostos podem também ser de grande importância como caracteres taxonômicos, auxiliando na identificação de espécies e compostos biologicamente ativos, com papel ecológico, na busca de novos compostos (WU et al., 2012) com fins terapêuticos.

***Mentha* x *piperita* L.**

O gênero *Mentha* apresenta cerca de 19 espécies e 13 híbridos naturais pertencentes a família Lamiaceae (TELES et al., 2013). O gênero inclui muitas espécies produtoras de óleo essencial utilizadas na perfumaria, cosméticos e indústrias farmacêuticas (TELCI et al., 2011) e é responsável por aproximadamente 2000 toneladas de óleo essencial, representando o segundo óleo mais importante depois do gênero *Citrus* (MUCCIARELLI et al., 2001). Além da utilização de espécies do gênero *Mentha* como base de alimentos e preparações de chá, os usos medicinais que remotam aos tempos antigos, incluem aplicações carminativas, antinflamatórias, antiespasmódicas, analgésicas e estimulantes (HENDRIKS, 1998; COWAN, 1999). As espécies de menta também são utilizadas contra náuseas, bronquite, flatulência, úlcera e problemas no fígado (FOSTER, 1990; TYLER, 1993; HENDRIKS, 1998; COWAN, 1999). Os óleos essenciais e extratos vegetais das espécies de menta apresentam atividades antimicrobianas (ISCAN et al., 2002), antioxidantes, citotóxicas (YADEGARINIA et al., 2006; GULLUCE et al., 2007; HUSSAIN et al., 2010; SINGH et al., 2011) e também revelaram propriedades inseticidas sendo considerados fontes para pesticidas ecologicamente correto (KUMAR et al., 2011).

A *Mentha* x *piperita* L., híbrido natural resultante do cruzamento de *M. aquatica* e *M. spicata,* espécie explorada na produção de terpenóides (MAFFEI et al., 1999) é conhecida como hortelã pimenta, menta e hortelã-apimentada. A espécie é considerada perene, apresenta forte odor pungente, semelhante ao da pimenta (*pepper*) de onde originou o nome específico 'piperita' (CHAUDHRY et al., 1957). A planta é nativa da Europa no Mediterrâneo, naturalizada nos norte dos Estados Unidos e Canadá, sendo cultivada em muitas partes do mundo (MCKAY; BLUMBERG, 2006).

A espécie é cultivada principalmente em áreas de clima temperado e tropical para a produção de óleo essencial nas folhas (MAFFEI; SACCO, 1987) que ocorre em órgãos especializados, os tricomas glandulares (TURNER et al., 2000). Na indústria alimentícia, as espécies *M.* x *piperita, M. arvensis* e *M. spicata*, são utilizadas como agentes flavorizantes e produção de alimentos e bebidas (ZHELJAZKOV et al., 2010) sendo consideradas espécies de maior importância econômica (TELCI et al., 2011). Seu óleo essencial, líquido de cor amarelo claro, odor forte e agradável, possui sabor aromático (CHAUDHRY et al., 1957), sendo um dos óleos essenciais mais produzidos e consumidos no mundo (ISCAN et al., 2002).

As folhas de hortelã pimenta são utilizadas pela população na forma de chá, em casos de má digestão, náuseas e problemas intestinais no aparelho digestivo (LORENZI; MATOS,

2002). A espécie *M.* x *piperita* tem sido utilizada como planta modelo para o estudo do metabolismo de terpenos (CROTEAU et al., 2005; RIOS-ESTEPA et al., 2008). Os principais monoterpenos encontrados no óleo essencial da *M.* x *piperita* são mentol, mentona, pulegona, limoneno, eucaliptol e acetato de mentila (MAFFEI; SACCO, 1987). O óleo essencial de hortelã pimenta apresenta mais de 200 componentes (LAWRENCE, 1988) sendo a maioria monoterpenos e a via biossintética inclui reações enzimáticas que conduz a formação do principal componente, o mentol, (RIOS-ESTEPA et al., 2008) considerado constituinte majoritário (ANSARI et al., 2000). Estudos com o óleo essencial da *M.* x *piperita* tem demonstrado apresentar atividade contra microorganismos, sendo sugerido o mentol como responsável pela bioatividade (ISCAN et al., 2002; AZUMA et al., 2003). O óleo essencial de *M.* x *piperita* também apresenta atividade larvicida e forte ação repelente contra mosquitos adultos (ANSARI et al., 2000). Além disso, o óleo essencial de hortelã pimenta também demonstra atividade genotóxica (LAZUTKA et al., 2001) e antiviral (MINAMI et al., 2003; SCHUHMACHER et al., 2003).

A proporção dos principais componentes voláteis identificados no óleo essencial da *M.* x *piperita* são mentol (33-60%), mentona (15-32%), isomentona (2-8%), eucaliptol (5-13%), acetato de mentila (2-11%), mentofurano (1-10%), limoneno (1.7%), β-mirceno (0.1–1.7%), β-cariofileno (2–4%), pulegona (0.5–1.6%) e carvona (1%) (SANG, 1982; PITTLER; ERNST, 1998; DIMANDJA et al., 2000; GHERMAN et al., 2000). O rendimento do óleo essencial das folhas da *M.* x *piperita* ocorre em torno de 1.2 - 3.9% em volume por massa de material seco (PICURIC-JOVANOVIC et al., 1997; BLUMENTHAL et al., 1998).

A composição química das plantas é conhecida por ser influenciada por fatores externos e alguns compostos podem ser acumulados em resposta à alterações ambientais (KOENEN, 2001) e condições de crescimento (FIGUEIREDO et al., 2008). A composição química do óleo essencial da *M.* x *piperita* varia com a maturidade, região geográfica, condições de processamento do óleo e variedade das espécies (CLARK; MENARY, 1981; ROHLOFF, 1999; GHERMAN et al., 2000; BLANCO et al., 2002; PINO et al., 2002; RUIZ DEL CASTILLO et al., 2003; XU et al., 2003). Diversos trabalhos na literatura demonstram a variação no conteúdo e composição química do óleo essencial da *M.* x *piperita* em diferentes condições de cultivo. Esses trabalhos correlacionam a otimização do cultivo da *M.* x *piperita* em diferentes condições de temperatura (BURBOTT; LOOMIS, 1967; FAHLEN et al., 1997), água (CHARLES, et al. 1990; MARCUM; HANSON, 2006; PEGORARO et al. 2010), luz (BURBOTT; LOOMIS, 1967; PEGORARO et al. 2010), nutrição (MAROTTI et al. 1994) e sazonalidade (MAROTTI et al. 1993), com a produtividade de biomassa vegetal e a biossíntese de compostos voláteis de elevado valor comercial. Entre as condições abióticas

que interefem no crescimento e metabolismo do vegetal a baixa diponibilidade de água causa o déficit hídrico e afeta o funcionamento normal, alterando o estado fisiológico e o equilíbrio da planta (GIMENEZ et al., 2005; XU et al., 2010; SHAO et al., 2008). Nas plantas medicinais e aromáticas, a menor disponibilidade hídrica no solo pode causar significantes alterações no acúmulo e composição do óleo essencial (PETROPOULOS et al., 2008) e influenciar a rota metabólica de biossíntese do óleo essencial promovendo reações de oxidação ou redução e assim, interferir no acúmulo e concentração de compostos específicos (CHARLES, et al. 1990).

Existe grande interesse em entender os fatores que afetam a biossíntese do óleo essencial de hortelã pimenta, a fim de aumentar o conteúdo de compostos comercialmente importantes, como mentol e mentona, e diminuir a presença de indesejáveis, pulegona e mentofurano (MAHMOUD; CROTEAU, 2002). Pulegona e metonfurano participam da rota metabólica de biossíntese dos monoterpenos e o composto pulegona é convertido a mentofurano ou mentona e então à mentol (MARCUM; HANSON, 2006). De acordo com estudos anteriores, a redução da mentona a mentol aumenta a qualidade do óleo essencial de hortelã pimenta (CLARK; MENARY, 1980).

Assim deve ser considerado que a composição química dos óleos essenciais das plantas é subordinada a variações quantitativas e qualitativas (HUSSAIN et al., 2010). Essas variações podem interferir nas atividades biológicas que são dependentes da composição química dos óleos essenciais, uma vez que, o material vegetal coletado em diferentes condições ambientais pode conter diferentes compostos ou novos compostos com outras bioatividades (ELOFF, 1999).

***Ocimum basilicum* L.**

O gênero *Ocimum* L., apresenta cerca de 150 espécies (JAVANMARDI et al., 2003), entre elas o manjericão doce (*Ocimum basilicum* L.). A espécie também é conhecida como manjericão, alfavaca, alfavaca-cheirosa, ou basílico sendo considerada uma das mais cultivadas em muitos países, pois representa fonte de matéria-prima para a indústria de óleos essenciais (JAVANMARDI et al., 2003; HUSSAIN et al., 2008). Seus extratos também são utilizados na produção de cosméticos e produtos farmacêuticos e pesticidas (UMERIE et al., 1998; KEITA et al., 2001; PASCUAL-VILLALOBOS; BALLESTA-ACOSTA, 2003).

O manjericão é uma erva anual, amplamente utilizada em muitos tipos de preparações culinárias em países do Mediterrâneo (SIFOLA; BARBIERI, 2006), contem flores roxas ou brancas, originalmente nativo da Índia e outras regiões da Ásia (KLIMANKOVA et al., 2008). De acordo com o aroma, o manjericão pode ser classificado em doce, limão, canela,

cânfora, anis e cravo (BLANK et al., 2004). As plantas de manjericão são utilizadas na culinária, como planta ornamental, medicinal e aromática, sendo o óleo essencial valorizado no mercado internacional pelo elevado teor do composto linalol (BLANK et al., 2004). Suas folhas são utilizadas secas ou frescas, como agente aromatizante em alimentos, produtos de confeitaria e bebidas (KOPSELL et al., 2005). Tradicionalmente a medicina popular tem empregado o uso da espécie em propriedades carminativas, estimulante e antiespasmódica (MAROTTI et al., 1996). O óleo essencial é utilizado nas indústrias alimentícias e perfumaria (PRASAD et al., 1986) e alguns dos seus componentes, como eucaliptol, linalol e cânfora, são conhecidos por apresentarem atividade biológica (MORRIS et al., 1979) como antibacteriana (PRASAD et al., 1985; ELGAYYAR et al., 2001) e inseticida (BOWERS; NISHIDA, 1980). Além disso, cânfora e eucaliptol sao compostos também relatados como agentes alelopáticos (RICE, 1979).

O elevado valor agregado ao óleo essencial de manjericão é devido a presença de fenilpropanóides, como eugenol, chavicol e seus derivados, ou terpenóides, como os monoterpenos linalol, metil cinamato e limoneno (SIFOLA; BARBIERI, 2006). Os componentes do óleo essencial de manjericão, eugenol, metil chavicol e linalol acumulam-se em estruturas especializadas, os tricomas glandulares (GANG et al., 2001). Os fenilpropanóides e terpenóides presentes no óleo essencial são sintetizados por rotas metabólicas diferentes. Os compostos chavicol, metil chavicol e eugenol são sintetizados a partir da rota do chiquimato, que produz o precursor aminoácido aromático fenilalanina (GANG et al., 2001; SANGWAN et al., 2001), enquanto os terpenóides são derivados da rota do mevalonato e/ou da deoxilulose (DXP) (MAHMOUD; CROTEAU, 2002).

O gênero *Ocimum* pertencente à família Lamiaceae é caracterizado por uma grande variabilidade de morfologia e quimiotipos (LAWRENCE, 1988). A facilidade de polinização cruzada levou ao surgimento de um grande número de subespécies, variedades e formas (GUENTHER, 1949). Diferentes quimiotipos tem identificado acessos de *O. basilicum* baseado na composição química do óleo essencial. Grayer et al. (1996) classificou cinco perfis de óleos dependentes da abundância dos seguintes compostos: (i) linalol; (ii) metil cavicol; (iii) eugenol; (iv) linalol e eugenol; (v) metil chavicol e metil eugenol. No mercado internacional os quimiotipos de *O. basilicum* são relatados com base na composição química do óleo essencial e localização geográfica (BOWES; ZHELJAZKOV, 2004). O manjericão Europeu tem como componentes principais, o linalol e metil chavicol, frequentemente considerado por conter sabor mais fino. O manjericão *Reunion* é o quimiotipo que possui maior nível de metil chavicol e o principal componente do quimiotipo tropical é o metil

cinamato. Outro tipo que possui elevado teor de eugenol é cultivado no Norte da África, Rússia e Europa Oriental e partes da Ásia (MAROTTI et al., 1996).

A literatura também mostra grande diversidade genética de *O. basilicum* quanto as características bioquímicas como antocianinas (SIMON et al., 1999) e compostos voláteis terpenóides e fenilpropanóides (IIJIMA et al., 2004). Além disso, também são consideradas diferenças nos cultivares quanto a morfologia relacionadas à altura das plantas, cor, tamanho e textura das folhas. Além das características morfológicas e aromáticas determinadas pelo genótipo, a composição química das plantas é muito influenciada pelas condições ambientais e técnicas agronômicas (PICCAGLIA et al., 1991; MAROTTI et al., 1996). A nutrição mineral é um exemplo a ser considerado, uma vez que, aplicações de nitrogênio mostraram aumentar a produção do óleo essencial em plantas aromáticas (RAM et al., 1995) assim como as diferentes fontes de nitrogênio, orgânico e inorgânico (ADLER et al., 1989; KANDEEL et al., 2002) que podem interferir no perfil do óleo essencial. Os principais componentes do óleo essencial de *O. balisicum* variam em relação a quantidades dependendo do quimiotipo da planta e também pelas técnicas de processamento do óleo essencial (PINO et al., 1994; GILL; RANDHAWA, 1996; MAROTTI et al., 1996). O método de secagem de plantas de manjericão para extração de óleo essencial e o cultivar (LACHOWIEZ et al., 1997) podem afetar o conteúdo e a qualidade do óleo essencial (GRAYER et al., 1996; YOUSIF et al., 1999)

Salvia deserta Shang

Salvia, maior gênero na família Lamiaceae, apresenta cerca de 900 espécies, amplamente distribuídas no mundo, na região do Mediterrâneo, África do Sul, América Central, América do Sul e Sudeste da Ásia. O nome *Salvia* (*sage*) é derivado de '*salvare*' originário do latin que significa 'cura' (TOPCU, 2006). Na Europa, particularmente em países do Mediterrâneo, infusões de várias espécies de *Salvia* são geralmente utilizadas na medicina popular, sendo que comercialmente as espécies mais utilizadas são *S. officinalis* e *S. triloba* (ULUBELEN; TOPCU, 1998; ULUBELEN, 2000). Algumas espécies de *Salvia* possuem importância econômica por serem utilizadas como agentes aromatizantes em perfumaria e cosméticos (WU et al., 2012) como *S. sclare* e *S. pratensis* (SALEHI et al., 2008) e outras são cultivadas para fins culinários (LU; FOO, 2002).

As espécies de *Salvia* demonstram atividades antioxidantes (TEPE, et al., 2006), antisséptica (DANIELA, 1993), antibacteriana (ULUBELEN, 2003), antifúngica (KOBAYASHI, 1987), antiviral (TADA, et al., 1994), citotóxica (LIN, 1989; ULUBELEN,

1999), carminativa, diurética e hipoglicemica (JIMENEZ, 1986). Keller (1978) listou mais de 60 doenças diferentes a qual espécies de *Salvia* possuem finalidade terapêutica.

As várias espécies de *Salvia*, conhecidas por possuir propriedades antimicrobianas, antioxidantes e anticâncer, são consideradas plantas populares utilizadas na medicina desde os tempos antigos (COWAN, 1999; GALI-MUHTASIB et al., 2000; BADISA et al., 2005;). As espécies de *Salvia* estudadas têm produzido uma série de metabolitos secundários, que têm atraído considerável atenção das comunidades científicas por apresentar um amplo espectro de atividades biológicas e novas estruturas (WU et al., 2012). Essas espécies são fontes ricas em compostos fitoquímicos incluindo flavonóides, sesquiterpenóides, diterpenóides, sesterpenes e triterpenes (LU; FOO, 2002; HASSANZADEH et al. 2011). A parte área dessas plantas contém flavonóides, triterpenóides e monoterpenos particularmente nas flores e folhas, enquanto os diterpenos ocorrem frequentemente nas raízes (TOPCU, 2006). No entanto, há registros na literatura de algumas espécies de *Salvia* Americana que contêm diterpenos na parte aérea e triterpenos e flavonas nas raízes (TOPCU, 2006).

Os diterpenos são metabólitos secundários estruturalmente mais diversificados isolados de espécies de *Salvia*. Vários compostos ativos como tanshinones, D (+) ácido láctico 3,4-dihidroxifenol, ácidos salvianólico (A-F) e ácido rosmarínico (LI, 1997; WANG et al., 2010) foram isolados e identificados nos últimos anos (TAYARANI-NAJARAN et al., 2013). Os diterpenos isolados a partir das espécies de *Salvia* demonstram atividades antioxidante, antibacteriana, antimutagênica, antiinflamatória e propriedades citotóxicas (AMARO-LUIZ et al., 1998) como por exemplo, o taxol, o cafestol, e kahweol substâncias que apresentam propriedades anticâncer (WU et al., 2012).

A *Salvia deserta* Shang é considerada planta herbácea, perene, geralmente encontrada em áreas de estepes, bordas de matas e margens de rios (ABDULINA, 1999). As plantas dessa espécie possuem 35-90 cm de altura, pequenas folhas e inflorescências roxas (PAVLOV et al., 1964). A espécie está distribuída na Ásia Central em regiões montanhosas, Cáucaso e Sibéria Ocidental (SOKOLOV, 1991) e a composição química de *S. deserta* contém ácidos orgânicos, alcalóides, vitamina C, taninos, flavonóides e quinonas (PAVLOV et al., 1964). Nas raízes, são identificadas quinonas e a parte aérea contém pequenas quantidades de óleo essencial (0,02%), com ação antimicrobiana (PAVLOV, 1964). As sementes secas contêm aproximadamente 23% de ácido graxo, utilizados na fabricação de vernizes. Na medicina popular, as partes aéreas são utilizadas para o tratamento de infecções intestinais e febre, enquanto as folhas e flores são utilizadas para problemas do coração. Seu fruto contém óleo, que é utilizado para cicatrização de feridas, taquicardia e disenteria. (PAVLOV, 1964).

A parte aérea de *S. deserta* contém triterpenóides como ursane, oleanano e derivados de *lupine* (SAVONA et al., 1987), enquanto diterpenos (royleanone, ferruginol, taxodione, etc.), derivados do ácido cafeico (ácido rosmarínico, ácido litospermico B, etc.) e o esteróide daucosterol (TEZUKA et al., 1998) são encontrados principalmente nas raízes.

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Apresentação dos Capítulos

Conforme estabelecido pelo programa de Pós-graduação em Ciências Biológicas (Botânica), expresso na portaria n° 01/2010-SPG/IBB/UNESP, os capítulos apresentados a seguir foram redigido na forma de um artigo científico. Para tal, foram seguidas as normas de formatação de periódicos científicos internacionais (A2, B1 – Comitê de Biodiversidade da Capes).

*Capítulo I – Manuscrito I**

*De acordo com as normas da revista *Environmental and Experimental Botany*

PEG-induced osmotic stress in *Mentha* x *piperita* L.: structural features and metabolic responses

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Abstract

The present study investigated whether osmotic stress induced by two polyethyleneglycol levels in a short time in peppermint (*Mentha* x *piperita* L.) changes the physiological parameters, leaf anatomy and ultrastructure and essential oil content and composition. Evaluations of water potential, relative water content, anatomy, ultrastructural features, gas exchange, chlorophyll fluorescence, biochemistry and essential oil content and composition were performed under two polyethyleneglycol levels (PEG 50 and PEG 100) in a hydroponic experiment. None of the tested morphological and physiological parameters of *M*. x *piperita* showed significant changes in plants exposed to PEG 50. These plants activated antioxidant defense systems and showed smaller morphological changes but no ultrastructural changes. By contrast, the osmotic stress caused by PEG 100 inhibited leaf gas exchange, reduced the essential oil content and changed the oil composition in the plants, including a decrease in menthone and an increase in menthofuran. However, PEG 100 increased the total soluble sugar content in plants, which indicates osmotic adjustments for preventing water loss. These plants also showed an increase in peroxidase activity, but this change was not sufficient to decrease the lipoperoxide level responsible for damaging the membranes of organelles. No significant changes were found in chlorophyll fluorescence, intercellular CO_2 concentration or water use efficiency in plants exposed to osmotic stress. Morphological changes were correlated with the physiological features evaluated, as plants exposed to PEG 100 showed collapsed cell areas, an increase in intercellular space, mesophyll thickening, and cuticle shrinkage. In addition, plants exposed to PEG 100 exhibited signs of cytoplasm degeneration, including smaller nuclei, morphological changes in plastids, and lysis of mitochondria and

organelles. In summary, our results reveal that PEG-induced osmotic stress in *M*. x *piperita* depends on the exposure time and intensity of osmotic stress applied. Plants exposed to higher PEG levels exhibited changes in physiological parameters, morphology and ultrastructural features, which modified essential oil content and quality and potentially also influenced growth.

Keywords: water deficit, *Mentha*, ultrastructure, enzymes antioxidant, gas exchange

1. Introduction

Plant growth and productivity are influenced by abiotic and biotic stresses. Plants are often exposed to stress conditions caused by temperature, salinity, water and nutrient availability and heavy metal toxicity (Shao et al., 2008). High temperature, light intensity and drought are among the most important environmental stresses affecting plant survival and crop productivity (Boyer, 1982). These environmental stresses trigger a wide variety of plant responses, ranging from changes in gene expression to altered cellular metabolism (Shao et al., 2008).

The use of polyethyleneglycol (PEG) is known to reduce the water potential (Michel, 1983) and induce plant water deficits (Perez-Alfocea et al., 1993; O'Donnell et al., 2013), causing physiological disorders and resulting in a lower water uptake and loss of cell turgor (Munoz-Mayor et al., 2012). Tissue dehydration affects plants at various levels of their organization (Yordanov et al., 2000), causing changes in water relations, biochemical and physiological processes, membrane structure and organelle morphology (Gaff, 1989; Stevanovic et al., 1992; Tuba et al., 1993; Sarafis, 1998). The reaction of a plant to water stress depend on the its intensity and duration of the stress as well as the plant species and its stage of development (Holtman et al., 1994; Jaleel et al., 2007; Jayakumar et al., 2007).

Certain plant responses may provide degrees of tolerance to osmotic stress (Amaya et al., 1999). Tolerant plants have developed strategies to cope with water deficits, including anatomical, morphological and metabolic mechanisms (Pereyra et al., 2012) that adjust their physiology and metabolism to accommodate osmotic stress (Bohnert and Sheveleva, 1998). One such strategy is the control of stomatal closure, the initial cell response against desiccation (Yordanov et al., 2000), which promotes transpiration reduction and water-saving by plants (Kaiser, 1987; Chaves, 1991). However, stomatal closure caused by osmotic stress reduces the CO_2/O_2 ratio in leaves and inhibits photosynthesis (Moussa, 2006), leading to further reductions in the photosynthetic electron chain and increased production of reactive oxygen species (ROS) (Candan and Tarhan, 2012). Consequently, to protect their cellular and

sub-cellular systems from the cytotoxic effects of ROS, plants activate antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) (Candan and Tarhan, 2012), to minimize oxidative stress.

Another tolerance response to water deficit is the accumulation of compounds, such as soluble sugars, proline and betaine (McCue and Hanson, 1990), that perform osmotic adjustments in cells and help plants resist drought to maintain sufficient turgor for growth (Carvajal et al., 1999) and tissue hydration.

Anatomical and ultrastructural changes are indicators of water deficit (Ciamporova, 1976; Shao et al., 2008). Low water conditions usually cause a reduction in cell volume (Guerfel et al., 2006) and increases in cell wall thickness (Guerfel et al., 2009) and cuticle thickness (Liakoura et al., 1999). Drought stress may also result in changes in the nuclei, cytoplasmic membranes, endoplasmic reticulum, mitochondria, dictyossomes, ribosomes (Ciamporova, 1976) and chloroplasts (Da Silvia et al., 1974). Several studies have been conducted to elucidate plant tolerance to osmotic stress in response to water deficit and to identify the mechanisms that allow plants to adapt to stress and maintain their growth, development and productivity; such studies also aid in the identification of resistant plants (Candan and Tarhan, 2012).

Mentha x *piperita* L. (peppermint), an important medicinal and aromatic plant belonging to the Lamiaceae family, is a natural hybrid resulting from crossing *M. aquatica* and *M. spicata* (Maffei et al., 1999). This specie is exploited for its production of terpenoids (Maffei et al., 1999) and is grown mainly for essential oil extraction (Maffei and Sacco, 1987). However, like most cultivated plants, its growth and yield can be affected by environmental constraints, such as water stress (Candan and Tarhan, 2012), salt stress (Oueslati et al., 2010) and osmotic stress. In the case of osmotic stress, different polyethyleneglycol levels alter the osmotic potential of a solution, generating a water deficit in plants. Osmotic stress may influence the growth and modify the content and quality of *M.* x *piperita*'s essential oil. We found no studies in the literature that characterize the influence of osmotic stress on primary metabolism and essential oil in association with morphology and ultrastructure in this specie. Thus, in the present study, we evaluated whether osmotic stress induced via two polyethyleneglycol levels in a short time (1) interferes with plant physiological parameters, (2) changes the anatomy and leaf ultrastructure, and (3) modifies essential oil content and composition. To this end, we evaluated the following variables: water potential and relative leaf water content, anatomical and ultrastructural features of the leaves, gas exchange, chlorophyll fluorescence, antioxidant enzymes levels, lipoperoxide levels, total soluble sugar content, and content and composition of essential oils.

2. Materials and methods

2.1. Plant material and location

Fertile branches were collected from adult plants grown in a bed at the Department of Botany, Biosciences Institute of UNESP, Botucatu City, Sao Paulo State, Brazil (22° 52' 20" S 48° 26'37" W). Vouchers were deposited in the Herbarium Irina Delanova Gemtchujnicov (BOTU) under number 027610.

For cuttings, stem fragments of peppermint (*M.* x *piperita)* measuring approximately 10 cm were placed in trays containing commercial substrate (Bioplant®, Nova Ponte, Minas Gerais, Brazil) and were maintained under humid conditions until rooting. After 25 days, the peppermint saplings were transferred to 5.0 L pots filled with complete Hoagland and Arnon's (1950) No. 2 nutrient solution. The pots were maintained in a greenhouse under mean maximum and minimum air temperatures of 31.5°C and 21.2°C, respectively, and a mean relative humidity of 75% until the harvest. The solutions were prepared using deionized water and were permanently aerated and renewed every week to minimize a pH shift and nutrient depletion.

2.2. Osmotic stress treatments

At 59 days after transplanting (DAT) to a hydroponic system, the plants were subjected to polyethyleneglycol (PEG-6000) treatments as an osmotic stimulator. Treatments consisted of a control (without PEG [PEG 0]) and two levels of osmotic stress. PEG 6000 was dissolved in the Hoagland solution at two levels: PEG 50 (50 g of PEG per 1000 mL) and PEG 100 (100 g of PEG per 1000 mL). The pots were arranged in a randomized design in a greenhouse with eight pots exposed to each of three treatments.

2.3. Leaf water status

The leaf water potential (Ψ_w) and relative water content (RWC) were measured before plants were exposed to osmotic stress (control condition) and then again 72 hours after PEG-induced stress. The Ψ_w was measured at 5:00 am (predawn) and 12:00 pm (midday) with a Dewpoint Potentiometer WP4-T (Decagon Devices Inc., Pullman, WA, US) and was expressed in 'MPa.'. For RWC measurement, the mature leaves were subjected to measurements of fresh weight (FW), dry weight (DW) and turgid weight (TW). RWC (%) was computed using the formula RWC (%) = [(FW - DW)/(TW - DW)] x 100.

2.4. Structural studies

2.4.1 Light microscopy (LM)

A fully expanded leaf was collected from each individual in each treatment (n=8) 24 hours after PEG administration. For analyses of glandular density, leaves were observed with a Leica M205C stereomicroscope, and the number of glandular trichomes in 1 mm^2 was calculated using the Leica Application Suite software (LAS).

For anatomical studies, leaf blade samples were fixed in FAA 50, dehydrated in alcoholic series and embedded in methacrylate resin (Gerrits, 1991). Cross sections (6 µm thick) were obtained with a rotatory microtome and stained with toluidine blue 0.05%, pH 4.7 (O'Brien, 1964). Permanent slides were mounted with Permount and examined with an Olympus BX 41 photomicroscopy equipped with a digital camera. Measurements were performed using the Cell^B Olympus-Imaging Software for Life Science Microscopy.

2.4.2 Scanning electron microscopy (SEM)

Leaf blade samples were collected 24 hours after PEG administration and fixed in 2.5% glutaraldehyde solution with 0.1 M phosphate buffer (pH 7.3) overnight at 4°C; they were then dehydrated in a graduated acetone series, critical-point dried, mounted on aluminum stubs, gold coated (Robards, 1978), and examined with a Fei Quanta scanning electron microscope at 12.5 kV.

2.4.3 Transmission electron microscopy (TEM)

Leaves were collected 24 hours after the treatments were administered and fixed in Karnovsky 2.5% in phosphate buffer 0.1 M (pH 7.3) for 24 hours at 5°C. The samples were post-fixed with 1% osmium tetroxide aqueous solution in the same buffer for 1 hour at 25°C and then dehydrated in a graduated series of acetone and embedded in Araldite resin (Machado and Rodrigues, 2004). Ultra-thin sections were stained with uranyl acetate and lead citrate (Reynolds, 1963). The samples were examined with a FeiTecnai™ transmission electron microscope at 80 kV.

2.5. Measurement of leaf gas exchange characteristics and chlorophyll fluorescence

After 48 hours of exposure to osmotic stress, the net photosynthetic rate (Pn, in µmol m^{-2} s^{-1}), stomatal conductance (g_s in mol m^{-2} s^{-1}), intercellular CO_2 concentration (C_i µmol CO_2 mol air^{-1}), transpiration rate (E mmol m^{-2} s^{-1}) and water use efficiency (WUE µmol CO_2 [mmol $H_2O]^{-1}$) of the plants' third fully expanded leaf were measured using an infrared gas analyzer (IRGA), the Li-Cor 6400 photosynthesis system (Li-Cor Inc., Lincoln, NE, US), between 09:00 am and 11:00 am. The CO_2 concentration reference used during evaluations was the level present in the environment, ranging from 380 to 400 µmol CO_2 mol^{-1}. The maximum quantum efficiency of PSII (Fv/Fm) was also determined after placing samples in darkness for

30 minutes with a PAM fluorometer Junior (Walz, Effeltrich, Germany). Leaf gas exchange and chlorophyll fluorescence were measured on the same day and under the same environmental conditions (25°C and PPFD of 1500 μmol m^{-2} s^{-1}).

2.6. Measurement of lipoperoxide levels and total soluble sugar content

Leaves from each treatment were collected 72 hours after treatment for lipid peroxidation (LPO) and total soluble sugars (TSS) analysis. The LPO assay was assessed according to the method described by Heath and Packer (1968). Samples were homogenized in a 5 mL solution containing thiobarbituric acid (TBA) 0.25% and trichloroacetic acid (TCA) 10% and incubated in a water bath at 90°C for 1 hour. After cooling, the homogenate was centrifuged at 10,000 g for 15 minutes at room temperature. Then, the supernatant collected from each sample was subjected to absorbance readings in a UV-visible spectrophotometer at 560 and 600 nm. For calculations, the malondialdehyde (MDA) molar extinction coefficient (155 mM cm^{-1}) was used. The TSS extraction was performed according to an adapted methodology from Garcia et al. (2006) using three replicates with 100 mg of leaf per treatment. The TSS was estimated colorimetrically using the phenol-sulfuric method (Dubois et al., 1956) with glucose (100 μg mL^{-1}) as a standard and expressed as milligrams per gram of fresh mass (mg g^{-1} FM).

2.7. Analysis of enzymatic antioxidant system

Seventy-two hours after the treatments were administered, leaves were collected for enzymatic antioxidant system analysis. Enzymatic extracts were obtained according to the method described by Kar and Mishra (1976). The assay of superoxide dismutase activity, (SOD [EC 1.15.1.1]), was conducted according to the method described by Beauchamp and Fridovich (1971). The reaction mixture was composed of 30 μL enzymatic extract, 50 mM sodium phosphate buffer pH 7.8, 33 μM nitroblue tetrazolium (NBT) + 0.66 mM EDTA (5:4), and 10 mM L-methionine + 3.3 M riboflavin (1:1), totaling 3.0 mL. Tubes were illuminated for ten minutes at 25°C, and NBT reduction to blue formazan was measured through absorbance readings in a UV–visible spectrophotometer at 560 nm. SOD activity was expressed as U mg^{-1} protein. In this case, one unit (U) represents the quantity of enzyme needed to inhibit the NBT reduction ratio by 50%. Peroxidase activity, (POD [EC 1.11.1.7]), was assayed according to the methods described by Teisseire and Guy (2000). The reaction mixture was composed of 30 μL diluted enzymatic extract (1:10 in the extraction buffer), 50 mM potassium phosphate buffer pH 6.5, 20 mM pyrogallol (benzene-1,2,3-triol) and 5 mM hydrogen peroxide (H_2O_2), totaling 1.0 mL. The reaction was carried out at room temperature for 5 minutes. Purpurogallin formation was measured in a UV–visible spectrophotometer at

430 nm, and its molar extinction coefficient (2.5 mM cm^{-1}) was used to calculate the specific activity, expressed as μmol purpurogallin.$minute^{-1}$ mg^{-1} protein. The catalase activity (CAT [EC 1.11.1.6]) assay was composed of 50 μL enzymatic extract, 950 μL 0.05 M sodium phosphate buffer pH 7.0 containing 12.5 mM H_2O_2, totaling 1 mL. After absorbance readings at 240 nm, the molar extinction coefficient of H_2O_2 (39.4 mM cm^{-1}) was used. The reaction was carried out at room temperature for 80 s. Readings were taken in a UV-visible spectrophotometer at 240 nm at 0 and 80 s. The enzyme activity was expressed as nmol consumed H_2O_2 $minute^{-1}$ mg^{-1} protein (Peixoto et al., 1999). The assessment of soluble protein levels from enzymatic extracts, necessary for calculating the specific activity of the studied enzymes, was performed according to the method described by Bradford (1976). Absorbance readings were conducted in a UV-visible spectrophotometer at 595 nm by using casein as the standard.

2.8. Analysis of essential oils

The aerial parts collected 72 hours after treatment were subjected to hydrodistillation in a Clevenger-type apparatus for 2 hours. The qualitative analysis of the essential oil compounds was performed on a gas chromatograph (GC) coupled to a mass spectrometer (MS) (GC–MS; Shimadzu QP5000) operating at an MS ionization voltage of 70 eV. The chromatography was equipped with a fused silica capillary column DB-5 (J and W Scientific; 30 m × 0.25 mm × 0.25 μm), and helium was used as the carrier gas. The following chromatograph conditions were used: injector at 240°C, detector at 230°C, gas flow 1.0 mL/minute, split 1/20, initial column temperature of 60–240°C at a rate of 3°C/minute, and a 1 μL injection of solution (1 mg of essential oil and 1 mL of ethyl acetate). The compounds were identified based on a comparative analysis of the acquired mass spectra with those stored in the GC–MS database of the system (Nist 62.Lib), in a previous study (McLafferty and Stauffer, 1989) and in retention indices (Adams, 2007), which were obtained from the injection of a mixture of n-alcanes (C_9H_{20}–$C_{25}H_{52}$, Sigma Aldrich, 99%) employing a column temperature program as follows: 60–240°C at a rate of 3°C/minute. Quantification (normalization area method) of the substances was carried out with a GC (Shimadzu GC-2010) equipped with flame ionization (GC–FID) and using a DB-5 (J and W Scientific; 30 m × 0.25 mm × 0.25 mm × 0.25 μm) capillary column. Helium was used as the carrier gas, and the temperature injector was at 240°C, the detector at 230°C, and gas flow at 1.0 mL/minute, split 1/20. The following chromatography conditions were used: 60–135°C at a rate of 5°C/minute, then 135–240°C at a rate of 8°C/minute and 60–240°C at a rate of 3°C/minute; 1 μL of solution was injected (1 mg of essential oil and 1 mL of ethyl acetate).

2.9. Statistical analysis

The overall effects of the treatments were determined by means of a one-way ANOVA followed by Tukey's test ($p<0.05$). Data were tested for normality and homogeneity of variances prior to analysis.

3. Results

3.1. Leaf water status

To observe the effect of osmotic stress in *M.* x *piperita* plants, we evaluated Ψ_w and RWC in control conditions (PEG 0) and 72 hours after the administration of PEG 50 and PEG 100 in the nutrient solution at predawn and midday. There was no difference in Ψ_w at predawn (Ψ_w pd) in plants subjected to PEG 50 and PEG 100 treatments, whereas the plants subjected to PEG 100 showed a reduction in Ψ_w at midday (Ψ_w md) of approximately 50% (Fig. 01). There was no difference in RWC in plants under PEG 50, but it was higher in PEG 100 plants compared to the control (Fig. 01).

3.2. Structural analysis

3.2.1 Leaf morphology

M. x *piperita* leaves are bifacial, amphistomatic and homobaric with glandular and non-glandular trichomes on both sides of the leaf surfaces (Fig. 02- A-E). The epidermis is uniseriate (Fig. 02- A, B) and consists of common cells that superficially exhibit a sinuous pattern (Fig. 02- C, D). On both sides of the leaf blade, the epidermal cells are covered with a thin cuticle. Two morphotypes of glandular trichomes were observed. The first shows a basal cell among the other cells of the epidermis and a large secretory head (Fig. 02- A, C). The second morphotype is composed of a basal cell, an unicellular short stalk and an oval head (Fig. 02- D, E). A higher density of glandular trichomes was observed on the abaxial surface of the leaf blade (Table 01). The stomata are arranged at the same level as epidermal cells or are slightly protruding (Fig. 02- C, D). The mesophyll is composed of a parenchyma palisade and three to four layers of spongy parenchyma (Fig. 02- A, B). Collateral bundles surrounded by well-defined endoderm were observed immersed in the mesophyll (Fig. 02- B). In the region of the midrib, the cortex is composed of one or two collenchyma layers on the abaxial surface, two to three layers on the adaxial surface (Fig. 02-F), and three to five layers of voluminous parenchyma cells with regular contours (Fig. 02- F, G.); the vascular system consists of xylem and phloem with the beginning of a cambial installation (Fig. 02- F, G).

Plants subjected to PEG 50 showed epidermal cells with more sinuous contours and slight cuticle shrinkage (Fig. 03- A, B) compared with control plants. However, no structural changes were observed under light microscopy (Fig. 03- C).

Under SEM, plants exposed to PEG 100 showed intense cuticle retraction and epidermal cell delimitation, which was less obvious when superficially viewed (Fig. 04- A, B). These leaves showed a higher trend of mesophyll thickness (146.19 µm) compared with the PEG 50 plants (139.85 µm) and control treatment plants (138.98 µm) (Table 1). In plants subjected to PEG 100, the area occupied by the intercellular spaces in the mesophyll was larger (592.18 μm^2) (Fig. 04- C) than in control plants (256.56 μm^2) (Fig. 01- A) (Table 1). Cell collapse regions were observed in several areas along the leaf mesophyll (Fig. 04- D). In plants subjected to higher osmotic stress, we observed more irregular contours (Fig. 04- E) in the parenchyma cortical cells of the midrib region compared with other treatments.

3.2.2 Subcellular features of leaf blade

In the leaf blade of control plants, the palisade and spongy parenchyma cells showed regular contours, smaller cytoplasms, developed vacuoles and large nuclei with evident nucleoli (Fig. 05- AC). In the cytoplasms, mitochondria, endoplasmic reticulum, chloroplasts, and dictyosomes were observed (Fig. 05- D). Chloroplasts were lenticular and ellipsoidal (Fig. 05- A, C) and had dense stroma, well-structured grana and widespread plastoglobules (Fig. 05- D); plastids exhibited starch grains (Fig. 05- B, C). These organelles were distributed mainly along the cell periphery (Fig. 05- A, B). In smaller caliber veins, the xylem consists of parenchyma cells and vessel elements; the phloem is composed of sieve tube elements and companion cells (Fig. 05- E) with thin walls, primary pit fields rich in plasmodesmata, and dense cytoplasms and mitochondria, endoplasmic reticulum, dictyosomes, polyribosomes and vesicles (Fig. 05- E, F).

Changes in subcellular features were not observed in plants exposed to PEG 50. In plants subjected to PEG 100, the parenchyma palisade and spongy cells of non-collapsed mesophyll regions showed changes in sinuous contours and the wall-folding regions (Fig. 06- A-C). Cytoplasm showed signs of degeneration, and the nuclei were decreased (Fig. 06- C). Dispersed oil droplets were observed in cytoplasm (Fig. 06- D). Mitochondria showed swelled cristae (Fig. 06- E); some of them showed signs of lysis (Fig. 06- E-G). Some chloroplasts exhibited an anomalous format (Fig. 06- F). Within chloroplasts, we observed denser stroma and a loss of the internal organization of thylakoid membranes (Fig. 06- E, F); starch grains and the presence of several plastoglobules were observed (Fig. 06-E, F). Within the vacuoles, flocculated content was observed (Fig. 06-C, G).

3.3. Measurement of gas exchange characteristics and chlorophyll fluorescence

Plants subjected to PEG 100 exhibited lower stomatal conductance (g_s) and transpiration (E) and photosynthetic rates (Pn) compared with control plants and PEG 50 plants (Fig. 07). These plants showed a 40% reduction in photosynthetic rate, whereas plants exposed to PEG 50 showed a 16% reduction, compared with control plants (Fig. 07). No difference was observed between the osmotic stress treatments in the intercellular CO_2 concentration (C_i) and water use efficiency (WUE) (Fig. 07). The values of Fv/Fm from plants exposed to osmotic stress (average 0.78 - 0.75 and PEG 50 - PEG 100) showed no significant differences compared with control plants (average 0.78) (Fig. 07)

3.4. Measurement of lipoperoxide levels and total soluble sugar content

LPO levels were higher in the leaves of plants exposed to osmotic stress (Fig. 08). The highest LPO level was observed in plants subjected to PEG 100, with an increase of 53% compared with the control. We observed an increase of 41% in TSS content in PEG 100 plants compared with control and PEG 50 plants (Fig. 08).

3.5. Analysis of enzymatic antioxidant system

Plants exposed to PEG 50 showed higher SOD and CAT activities when evaluated at 72 hours after the administration of PEG (Fig. 09). Plants subjected to PEG 100 showed no differences in SOD or CAT activities compared with control, but POD activity increased by approximately three-fold (Fig. 09).

3.6. Analysis of essential oils

Control and PEG 50 plants showed higher essential oil content (1.32% and 1.30%, respectively) compared with PEG 100 plants (0.87%) (Table 2). Twenty compounds identified represent 99% of the essential oil (data not shown). The major compounds found were menthone (39.4%), menthofuran (32.6%), menthol (15.3%) and pulegone (5.07%) (Table 2). Eucalyptol and limonene (data not shown) were also detected (2.6% each). There was a 36% reduction in menthone in plants subjected to PEG 50 and a 53% reduction in plants treated with PEG 100. The osmotic stress caused by PEG 100 increased the menthofuran percentage by 25%, whereas menthol and pulegone were not affected by treatments.

4. Discussion

In the present study, *M.* x *piperita* plants exposed for 72 hours to osmotic stress induced by two levels of PEG 6000 showed structural, cellular and physiological changes compared with plants grown in control conditions. The results indicated that osmotic stress responses were dose dependent, as plants subjected to PEG 50 maintained structural features and metabolic functions similar to those of control plants. Plants exposed to PEG 100 showed anatomical changes and ultrastructural damage, which are consistent with the low leaf water potential, gas exchange reduction, and increases in total sugars and the activity of antioxidant enzymes. In addition, we observed that the increased antioxidant enzyme activity was not sufficient to prevent the degradation of the membranes. Our results also indicate that osmotic stress caused by PEG 100 influenced the essential oil content and composition of *M.* x *piperita.*

Plants subjected to PEG 50 were more tolerant to exposure to osmotic stress because they were able to maintain the photosynthesis rate and stomatal conductance, showing high intercellular CO_2 concentrations and transpiration rates, which are indicative of a normal flow of water and root uptake, as observed in control plants.

In plants exposed to PEG 100, the leaf water potential showed a higher reduction in response to osmotic stress during midday. Under control conditions, with increasing temperature and decreasing relative humidity, the transpiration rate exceeds the water uptake by the roots and causes a water deficit in plants; thus, water potential during midday is more negative, and this effect is accentuated under stress conditions (Gimenez et al., 2005; Kudoyarova et al., 2013). These plants recovered from the deficit produced by transpiration during daylight hours, showing an increase in leaf water potential during predawn and maintaining tissue hydration, as observed in RWC. Predawn is characterized by a lower water loss by transpiration, as stomata are partially closed and the plant continues to uptake water until complete rehydration, an equilibrium state are achieved overnight (Gimenez et al., 2005; Kudoyarova et al., 2013).

M. x *piperita* plants subjected to PEG 100 showed a 40% reduction in photosynthetic rate and lower transpiration, which is likely attributable to stomatal closure partially in response to osmotic stress. According to Kudoyarova et al. (2013), plants in water deficit conditions perform osmotic adjustment mechanisms to maintain the water content in tissues, such as the partial stomatal closure and decreased transpiration observed in our study. According to Chaves et al. (2003), plants in water deficit conditions show short-term responses such as stomatal closure, decreased carbon assimilation and osmotic adjustments.

Additionally, we showed that osmotic stress did not affect photochemical activity, as PEG-treated plants showed similar Fv/Fm compared with control plants, confirming that the photochemical apparatus is resistant to applied osmotic stress. Similar results were found by

Guóth et al. (2008) and Silva et al. (2010), who also did not observe damage to the photochemical apparatuses in plants exposed to PEG.

Plants subjected to PEG 100 showed a higher TSS content than PEG 50 and control plants. Solute accumulation in the cytoplasm is a mechanism that plants use during water deficit to adjust to low water availability (Bohnert and Jensen, 1996; Bacelar et al., 2009; Kudoyarova et al., 2013), avoiding dehydration and tolerating a low tissue water potential (Chaves et al., 2003). We observed this response in RWC.

Plants exposed to PEG 50 had a similar photosynthetic rate to that of the control, which may be related to the activity of antioxidant enzymes. The SOD and CAT activity levels were higher in the PEG 50 plants than in PEG 100 plants. This response demonstrates that plants exposed to PEG 50 activated protective mechanisms against the presence of free radicals in an attempt to maintain normal metabolic functions. Among the antioxidant enzymes, SOD is the primary line of defense against ROS (Blower et al., 1992) and eliminates superoxide radicals, producing O_2 and H_2O_2. H_2O_2 is harmful to chloroplasts, nucleic acids and proteins and is subsequently eliminated by the action of CAT and POD enzymes and other non-enzymatic antioxidants (Fatima and Ahmad 2004; Srivastava et al., 2010).

The POD enzyme, which removes H_2O_2, also contributes to the defense system of *M*. x *piperita* against osmotic stress. Plants subjected to PEG 100 showed higher POD activity. Similar results observed by Oueslati et al. (2010) observed an increase in POD activity among plants exposed to osmotic stress, and Lechno et al. (1997) reported an increase in the activites of the enzymes, CAT and glutathione reductase in plants subjected to salt stress. The same authors did not verify an increase in SOD activity, a result also found in plants subjected to PEG 100. Activation of SOD and CAT may have occurred before the evaluation period, as POD activity was higher in these plants. We also suggest that SOD activity may have been impaired in these plants because damages were observed in the chloroplasts and mitochondria, which are SOD isoform reaction centers (Zelko et al., 2002; Munoz et al., 2005).

Candan and Tarhan (2012) demonstrated that SOD activity in *M. pulegium* plant was positively correlated with the severity of osmotic stress and exhibited maximal activity at the end of evaluation period. According to Shao et al. (2008), reactions of plants to drought depend on the intensity and duration as well as plant species and stage of development. Moreover, Candan and Tarhan (2012) emphasized that a positive antioxidant response to abiotic stress is a symptom of tolerance, and the opposite behavior is therefore evidence of sensitivity. In our study, *M.* x *piperita* showed tolerance depending on the osmotic stress level applied.

In addition, the antioxidant enzymes activity showed a positive correlation with the intensity of applied stress, as PEG 50 plants showed higher SOD and CAT activities and lower LPO levels. Lipid peroxidation is represented by malondialdehyde accumulation (MDA) and is indicative of oxidative damage in cells (Ennanjeh et al., 2009; Zhang et al., 2011). Low concentrations of MDA have been associated with drought tolerance in some species (Moran et al., 1994; Sairam et al., 2000). Moreover, the membranes of plants exposed to PEG 100 showed oxidative damage, as demonstrated by the increase in LPO levels. The higher POD activity was not sufficient to control damage caused by oxidative stress. *M. pulegium* subjected to osmotic stress for 8 days showed an increased LPO level with time, which was higher in plants subjected to severe stress than those subjected to moderate stress (Candan and Tarhan, 2012). The increase in LPO levels affects the growth and productivity of plants exposed to abiotic stresses (Ennanjeh et al., 2009; Zhang et al., 2011).

Changes in the structural characteristics of the leaves and other anatomical and subcellular changes caused by osmotic stress were observed in *M.* x *piperita* exposed to PEG and were more evident in plants subjected to PEG 100. We observed immediate changes, such as cell collapse, cuticle shrinkage and the degeneration of cytoplasmic organelles. The time of plants' exposure to PEG treatment was not sufficient to ensure the formation of cells and tissues under osmotic stress conditions and thus enable the identification of ontogenetic changes in leaves. Different effects of stress caused by water deficit in the anatomy and ultrastructure of leaves and other tissues in several plant species have been reported (Paakkonen et al., 1998; Chartzoulakis et al., 1999; Dekov et al., 2000; Guerfel et al., 2009; Zaharah and Razi, 2009; Ennajeh et al., 2010).

Plants subjected to PEG 50 exhibited smaller changes in epidermal cells, which showed mild shrinkage and thickening of the cuticle and increased cell contour sinuosity. No changes in subcellular features were observed.

In plants exposed to PEG 100, anatomical and ultrastructural changes were more intense. The cuticle was found to be retracted, the area occupied by intercellular spaces increased and consequently increased the mesophyll thickness, and collapse cell areas occurred on the leaf mesophyll. Sam et al. (2003) reported that salt stress affected the organization of parenchymatic cells, increased intercellular spaces and changed the format of cells from tomato plants. These authors concluded that such changes depended on the NaCl level used and were associated with the tolerance of cultivars to salt stress.

Changes in the thickness of mesophyll in plants exposed to osmotic stress may have been caused by the increased area of intercellular spaces of spongy parenchyma found in PEG 100

plants. Similar results were observed by Rajabpoor et al. (2014), who also noted that the length of palisade parenchyma cells remained unchanged.

According to Bosabalidis and Kofidis (2002), another anatomical change that occurs in plants subjected to water deficit is a size reduction of epidermal and mesophilic cells. Small size and straight, not sinuous, walls contribute to resistance against cell collapse in response to water deficit (Oertli et al., 1990; Bosabalidis and Kofidis, 2002). However, although parenchymatic cells showed sinuous contours in *M.* x *piperita* plants subjected to PEG 100, there was no significant reduction in cell size, which may be associated with the formation of collapsed areas in the mesophyll. The presence of these collapsed areas in the leaf mesophyll may have influenced *M.* x *piperita* metabolic processes, causing a decrease in CO_2 uptake area and interfering with gas exchange and consequently with the growth of the plants.

Regarding subcellular features, the parenchyma cells of *M.* x *piperita* mesophyll exposed to PEG 100 showed signs of cytoplasm degeneration, including smaller nuclei, morphological changes in plastids and lysis of mitochondria and organelles.

We observed that plants exposed to PEG 100 exhibited swelled cristae. Morphological changes in mitochondria appear to be a common feature in plants under different stresses (Ciamporova, 1976; Sam et al., 2003; Rodrigues et al., 2014) and may indicate changes in the mitochondrial energy status resulting from decreases in ATP levels (Kandasamy and Kristen, 1989; Pareek et al., 1997) and increases in the levels of ROS (Li et al., 2012). Ciamporova (1976) and Ciamporiva (1977) observed that changes in mitochondria caused by PEG were dependent on the time of exposure to osmotic stress; up to 24 hours, mitochondria showed irregularly shaped, small cristae, dense and fusiform inclusions and some fragmented membranes. After 48 hours of exposure to osmotic stress, both mitochondria and plastids were smaller and denser and were difficult to identify.

In the present study, we observed that the chloroplasts of plants treated with PEG 100 showed denser stroma and a loss of organization in the internal membrane system as well as the presence of more plastoglobules, which may have been associated with the decreased photosynthetic rate observed in these plants. Similar reports have found changes in plastid format (Lechno et al., 1997; Paakkonen et al., 1998; Sam et al., 2003; Xu et al., 2006; Li et al., 2012), destruction of the plastid envelope (Yamane and et al., 2003) and altered thylakoid membranes (Giles et al., 1974; Silva et al. 1974; Paakkonen et al., 1998; Yamane et al., 2003; Zang et al., 2005; Xu et al., 2006; Li et al., 2012; Lima et al., 2013) in plants under different stresses.

The abundance of plastoglobules may be associated with increased oxidative stress in the photosynthetic apparatus (Paakknonen et al., 1998; Sam et al., 2003; Austin et al., 2006).

Plastoglobules are lipoprotein particles within chloroplasts that may act to protect against free radicals (Austin et al., 2006). Generally, these particles are produced during the regulation of lipid metabolism in response to oxidative stress and during senescence (Austin et al., 2006); their number increases when plants are exposed to low water availability (Paakkonen et al., 1998).

Both mitochondria and chloroplasts are sites of activity antioxidant enzymes (Alscher et al., 2002; Vyas and Kumar, 2005; Asada, 2006) and ROS generation in plants. ROS production may have a deleterious effect on the photosystem and thylakoid membranes (Piller et al., 2014.), leading to lysis in organelles and consequently to cell death (Isaiah and Oliveira, 2012). In fact, in the present study, we observed organelles in the process of lysis in the cytoplasm of parenchymatic cells of *M.* x *piperita* leaf blades exposed to PEG 100. To limit damage, plants increase the production of antioxidants, such as tocopherol (Piller et al., 2014), carotenoids, and vitamin E (Austin, 2006), and the activity of antioxidant enzymes (Alscher et al., 2002). Changes in mitochondria and chloroplasts found in *M*. x *piperita* of the PEG 100 treatment within 24 hours represent osmotic stress signals that are associated with the lowest CO_2 assimilation rate and antioxidant enzyme activity found in these plants. However, SOD activity in these plants did not increase as expected; thus, we suggest that changes observed in the ultrastructure of chloroplasts and mitochondria may have been a result of excess superoxide radicals.

Essential oil content decreased with the increase of osmotic stress intensity after 72 hours of treatment. This result is in agreement with studies in other aromatic species, such as *Cymbopogon nardus*, *Cymbopogon pendulus* (Singh-Sangwan et al., 1994) and *Matricaria chamomila* (Razmjoo et al., 2008), exposed to lower water availability.

In plants subjected to PEG 100, the decrease in essential oil content is associated with the influence of storage in glandular trichomes in collapsed areas of the leaf blade. In Lamiaceae species, glandular trichomes are responsible for biosynthesis, secretion and essential oil accumulation (Gershenzon et al., 1989; McCaskill et al., 1992; Turner et al., 2000). The presence of glandular trichomes observed in our study was also reported by Turner et al. (2000), Martins (2002), and Maffei (2010). In addition, osmotic stress may have affected the accumulation of essential oil due to the assimilation of CO_2, which was lower in these plants. According to Delfine et al. (2005), the reduction of photosynthesis due to water deficit may decrease monoterpene production, which depends on CO_2 acquisition and the formation of intermediate photosynthesis (Loreto et al., 1996).

Our results showed *M.* x *piperita* with limonene, eucalyptol, menthone, menthol, menthofuran and pulegone compounds, totaling 97.6% of oil composition and representing the major

compounds. A similar composition was found by Bufalo et al. (2014) but with menthol as a major compound in older plants. In the present study, *M.* x *piperita* exhibited higher menthofuran amounts, which may be related to the age of plants. Limonene, pulegone and menthofuran proportions decrease with plant development, whereas eucalyptol, menthol and neomenthol increase (Gershenzon et al., 2000). Usually, high-quality *M.* x *piperita* oil should contain high amounts of menthol, moderate amounts of menthone and low levels of menthofuran and pulegone (Mahmoud and Croteau, 2003).

Under osmotic stress conditions, the essential oil composition of *M.* x *piperita* showed changes in the production of oxygenated monoterpenes, menthone and menthofuran. Plants exposed to osmotic stress (PEG 50 and 100) showed a reduction in menthone, and only plants from the PEG 100 treatment had an increase in menthofuran. Similar results were observed by Charles et al. (1990) in *M.* x *piperita* subjected to osmotic stress, wherein different intensities of stress at the beginning of plant development were evaluated. The authors observed an increase in menthone levels after one week of exposure to stress and a subsequent reduction of menthol at 21 days in the development cycle. Furthermore, the authors observed that osmotic stress decreased pulegone levels and increased menthofuran, suggesting its influence on oxidative and reductive transformations of pulegone. Our results indicate the oxidation of pulegone to menthofuran and the consequent reduction of menthone in plants from the PEG 100 treatment. Other studies involving abiotic stresses showed that light, temperature and humidity conditions also promote the accumulation of pulegone and menthofuran (Burbott and Loomis, 1967; Clark and Menary, 1980). Pulegone is a central intermediate in the biosynthesis of menthol, and depending on environmental conditions, this metabolite may be reduced to menthone and then produce menthol through the action of pulegone reductase enzyme or be oxidized to menthofuran by menthofuran synthase (Mahmoud and Croteau, 2003). Therefore, stress generated by PEG 100 altered the essential oil metabolic route of *M.* x *piperita* by influencing menthofuran synthesis, a condition that interferes with the quality of the essential oil.

This study shows that plants exposed to PEG 50 maintained metabolic functions similar to those of the control treatment without changes to gas exchange or structural characteristics. The increase in antioxidant enzyme activity reduced the presence of free radicals and protected membranes, including chloroplasts and mitochondria, which are important reaction centers of primary and secondary metabolism. These responses represent the tolerance of plants to this level of applied PEG. PEG 100 application reduced the leaf water potential, decreased gas exchange, increased total sugar content and activated the antioxidant defense system, changes that were not sufficient to stop the degradation of membranes. This condition

changed structural characteristics, caused damage at the cellular level, and reduced essential oil content and quality. These results suggest that responses to osmotic stress are related to intensity and to the applied dose and its duration. We conclude that the changes in physiological parameters, leaf structure and ultrastructure, and essential oil content and quality in *M.* x *piperita* depend on the duration and intensity of the osmotic stress applied.

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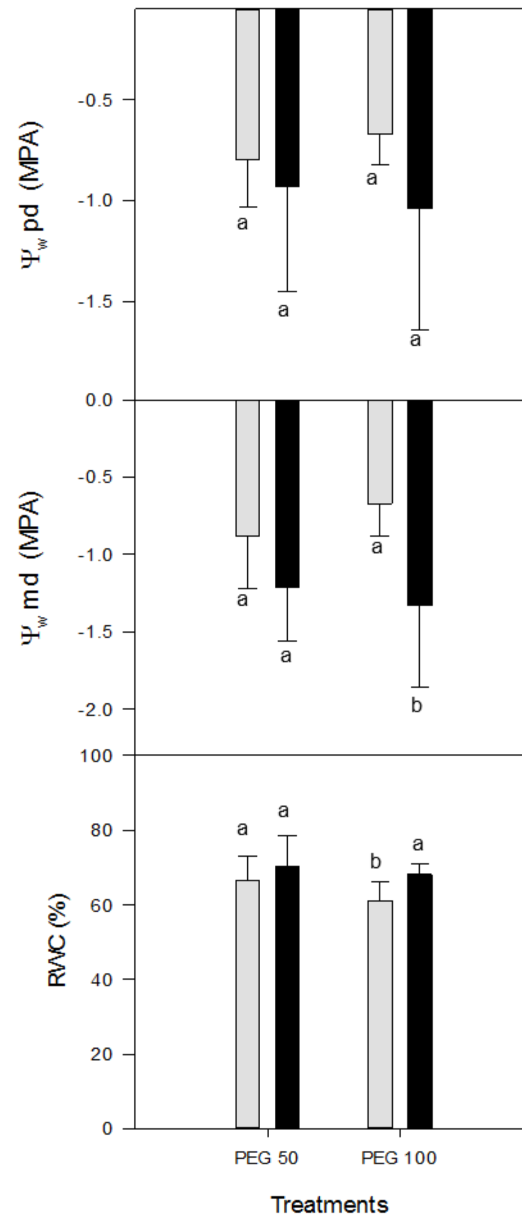

Fig. 1. Effects of osmotic stress on leaf water potential at predawn (Ψ_w pd) and midday (Ψ_w md) (MPa) and the relative water content (RWC) (%) of *Mentha* x *piperita* L. plants before exposure to osmotic stress (gray bar) and 72 hours after treatments administration (PEG 50 and PEG 100) (black bar). Data are means ± SD of eight samples. Different letters indicate a significant difference ($p < 0.05$) according to Tukey's test.

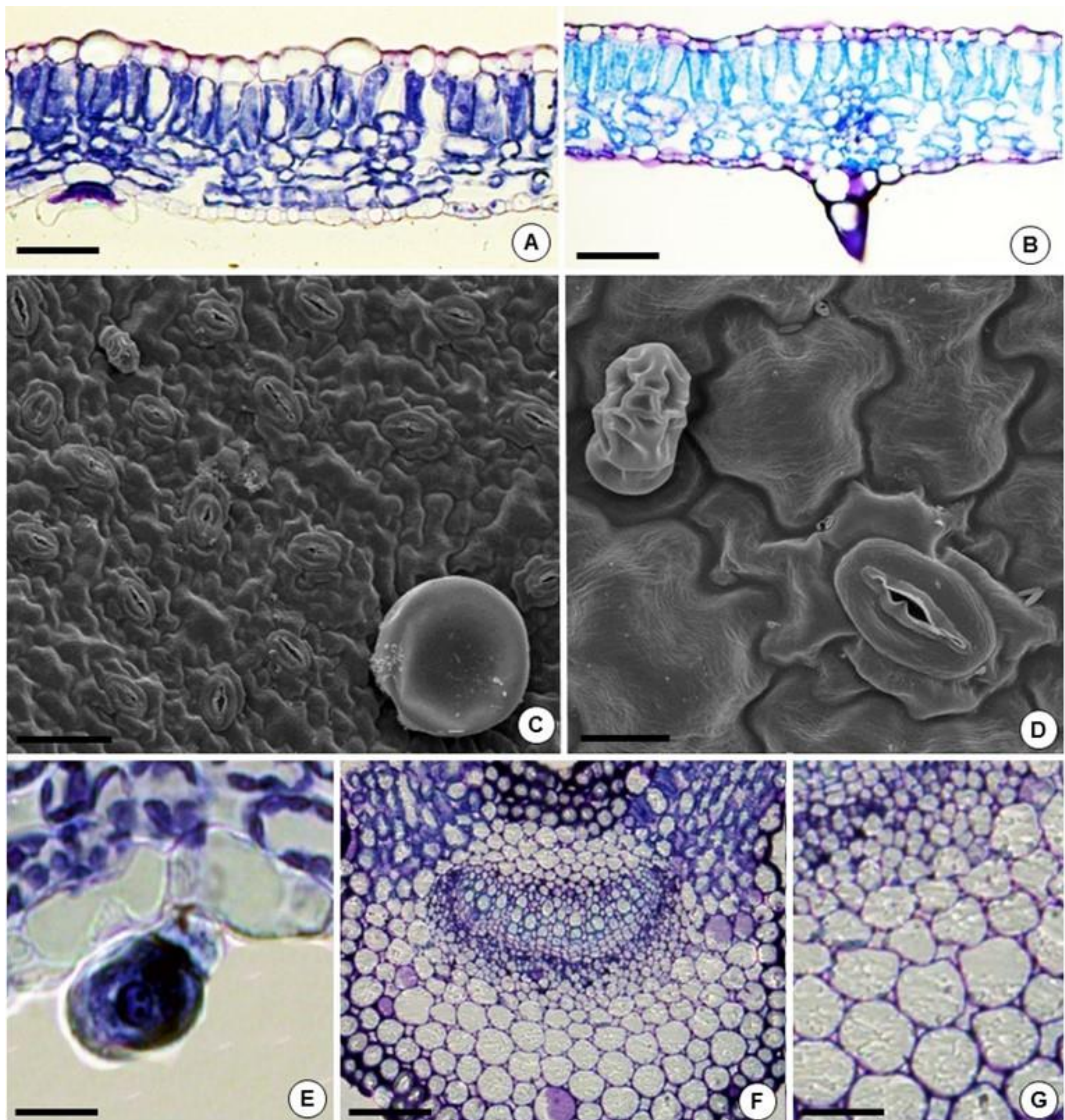

Fig. 2. Leaf blade of *Mentha* x *piperita* L. subjected to PEG 0 (control treatment). A, B, E-G. Photomicrographs. C, D. Electron micrographs (SEM). A, B. Sections showing uniseriate epidermis and dorsiventral mesophyll. Observe the glandular trichomes with large secretory heads in A and the non-glandular trichomes in B. C. Front view of the epidermis on the abaxial surface of the leaf blade showing stomata and glandular trichomes. D. Front view of the epidermis of the adaxial surface of the leaf blade showing a slightly striated cuticle, stomata and glandular trichomes. E. Detail showing glandular trichomes with secretory oval heads on the abaxial surface of the leaf. F. General view of the midrib showing cortex consisting of parenchymatic and collenchymatic cells and a vascular system composed of primary xylem and phloem. G. Details of the cortical parenchyma of the midrib showing turgid cells with regular contour. Bars: A, B = 40 µm; C = 80 µm; D, E, L = 25 µm; F = 50 µm.

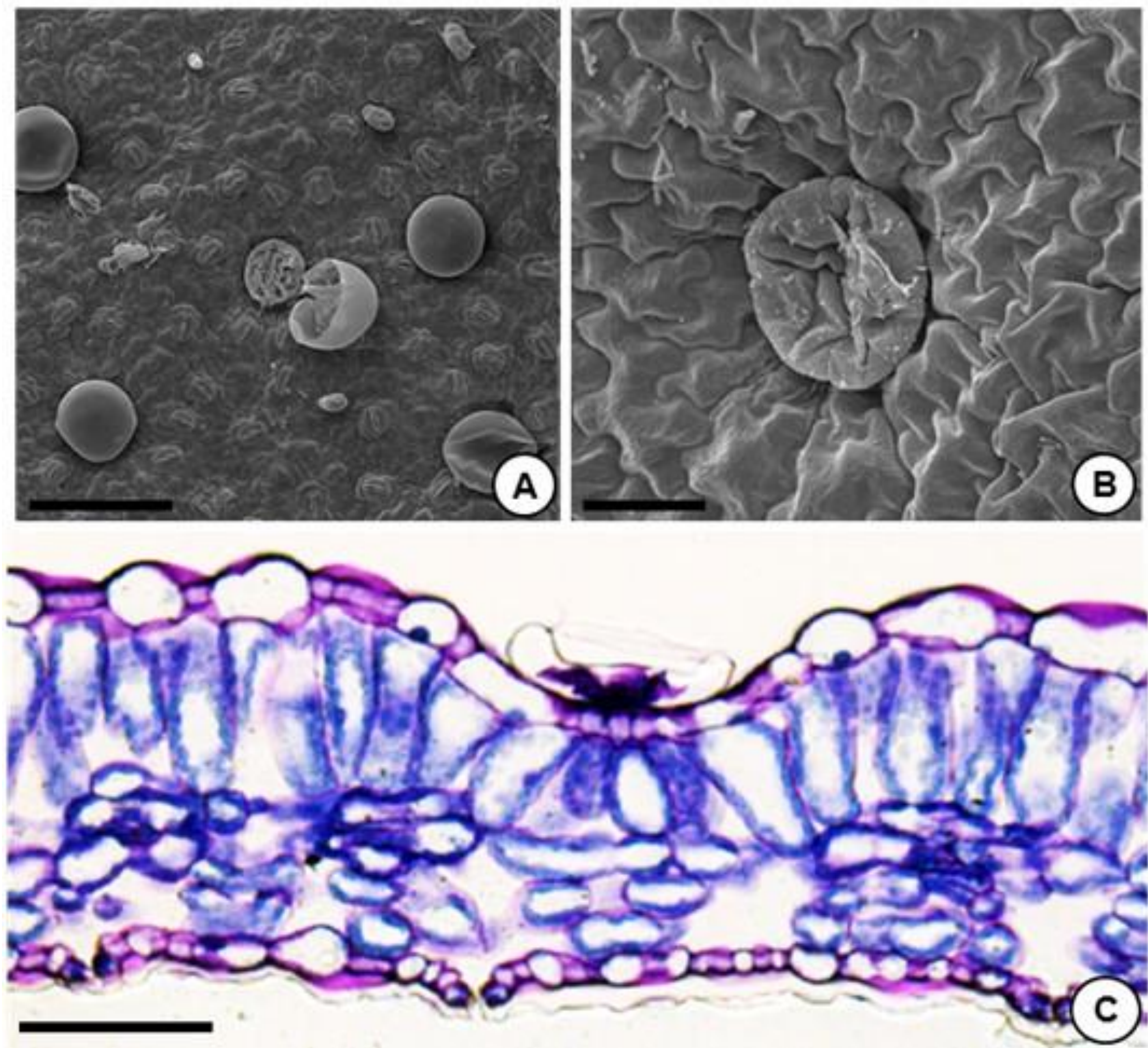

Fig. 3. Leaf blade of *Mentha* x *piperita* L. subjected to PEG 50. A, B. Electron micrographs (SEM). A. Front view of the epidermis on the abaxial surface of the blade showing stomata and glandular trichomes. B. Front view of the epidermis of the adaxial surface of the blade showing glandular trichomes and common epidermal cells with sinuous contour. C. Photomicrograph of a cross section of the leaf blade showing uniseriate epidermis with glandular trichomes and stomata, dorsiventral mesophyll and small vascular bundles. Bars: A = 90 µm; B = 40 µm; C = 30 µm.

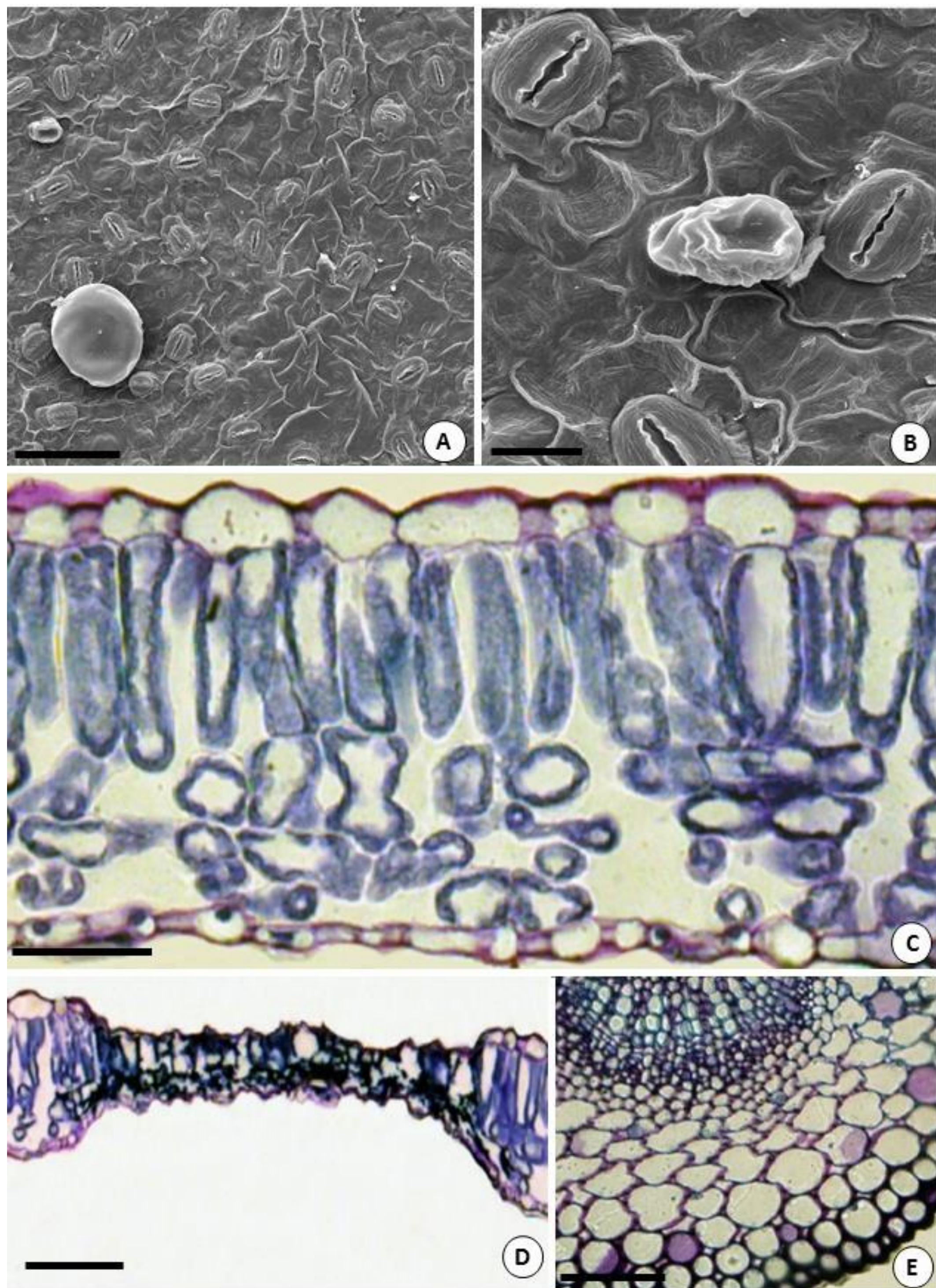

Fig. 4. Leaf blade of *Mentha* x *piperita* L. subjected to PEG 100. B. Electron micrographs (SEM). C-E. Photomicrographs A, B. A. Front view of the epidermis on the abaxial surface of the leaf blade showing stomata and glandular trichomes. Observe the retraction of the cuticle. B. Epidermis on the adaxial surface of the leaf blade showing stomata, glandular trichomes and retraction of the cuticle. C. Cross section of an intact region of leaf blade showing uniseriate epidermis and dorsiventral mesophyll. D. Cross section showing a collapsed region of the leaf blade. E. Midrib showing the epidermis, cortex and vascular system. Notice the irregular contours of the cortical parenchyma cells. Bars: A = 90 μm; B = 25 μm; C = 30 μm; D = 40 μm; E = 50 μm.

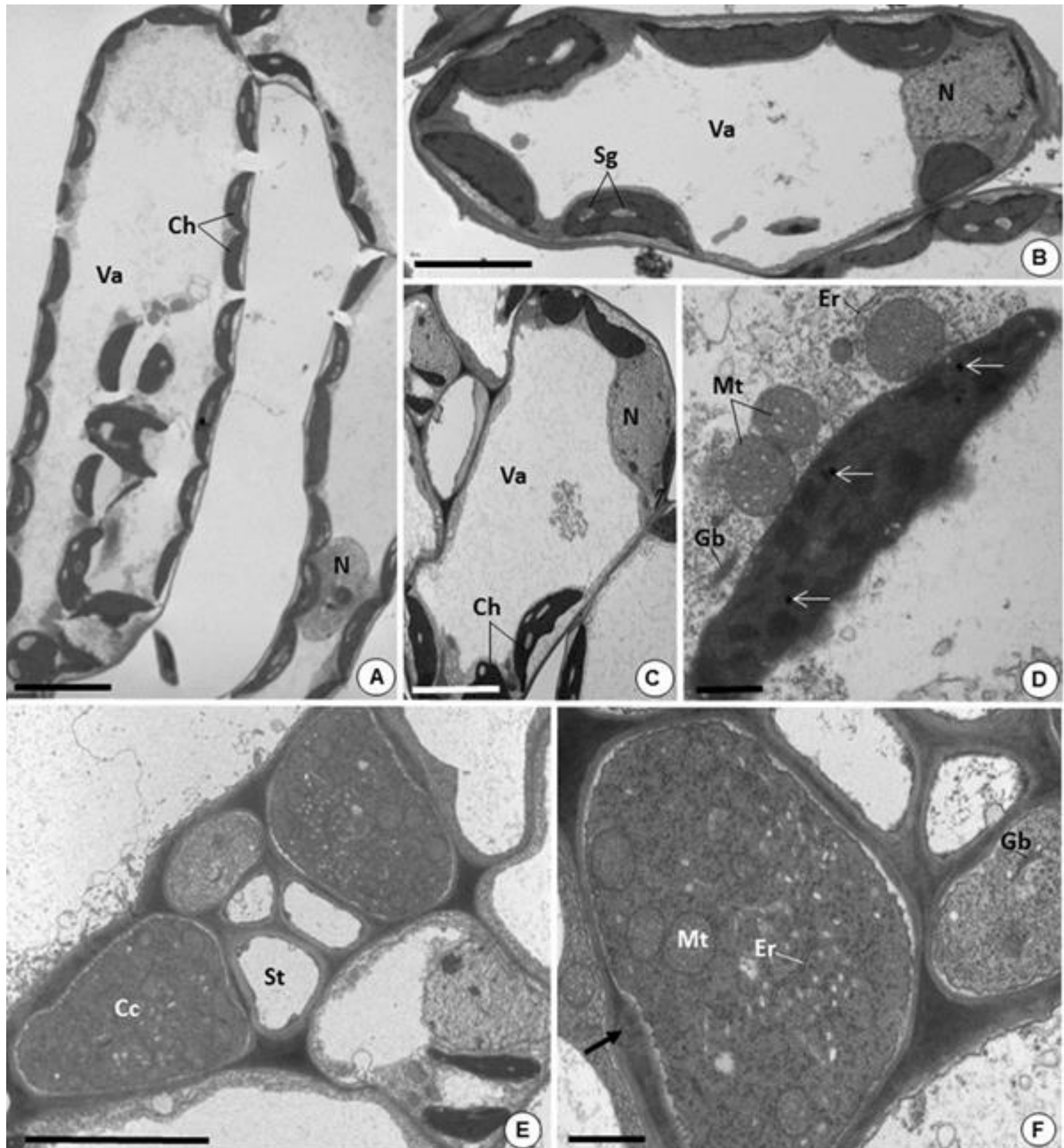

Fig. 5. Electron micrographs (MET) of *Mentha* x *piperita* L. leaf blades subjected to PEG 0 (control treatment). Palisade parenchyma cells showing chloroplasts (Ch) with lenticular shape preferably distributed in the cell periphery. N: nuclei. Va: vacuole. B, C. Palisade and spongy parenchyma cells, respectively, showing chloroplasts (Ch) with starch grains (Sg) and structured grana. N: nuclei. Va: vacuole. D. Detail of parenchyma cell showing chloroplasts with structured grana and plastoglobules (white arrows), mitochondria (Mt), Golgi bodies (Gb) and endoplasmic reticulum (Er). E. General view showing sieve tube elements of the phloem (St) and companion cells (CC). F. Detail showing companion cells with dense cytoplasm rich in polyribosomes, mitochondria (Mt), endoplasmic reticulum (Er), Golgi bodies (Gb) and vesicles. The arrow indicates plasmodesmata. Bars: A = 10 μm; B, D, E = 5 μm; C, F = 1 μm.

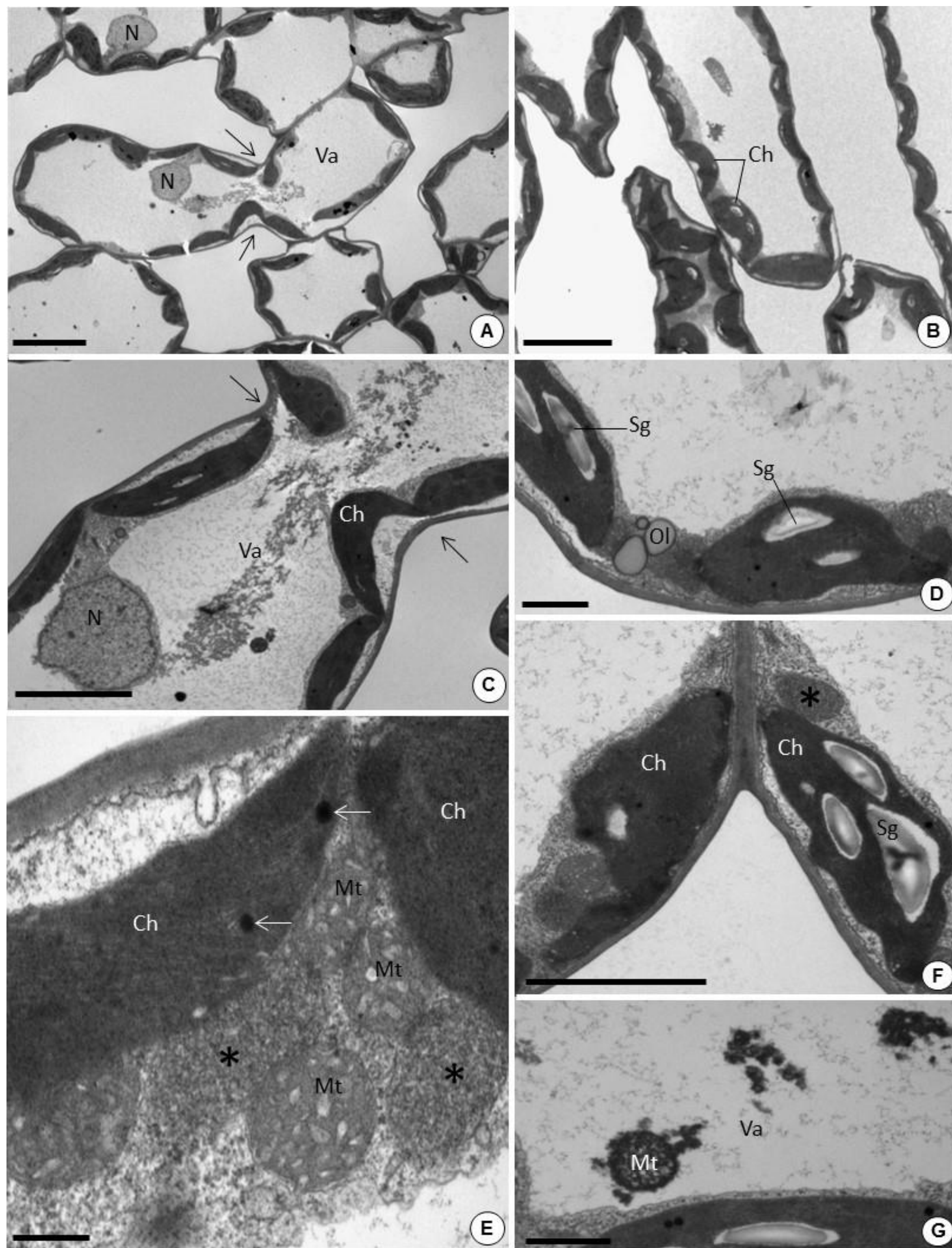

Fig. 6. Electron micrographs (MET) of *Mentha* x *piperita* L. leaf blades subjected to PEG 100. A, B. General aspect of the chlorophyll parenchyma showing cells with wall-folding regions (black arrows), chloroplasts (Ch) with ellipsoid format and smaller nuclei (N). Va: vacuole. C. Detail of a parenchyma cell showing nuclei (N), smaller chloroplasts (Ch) with ellipsoid shape and vacuoles (Va) with flocculated content. Black arrows indicate areas of cell constriction formed by the folding of the wall. D. Detail showing chloroplasts with electron-dense stroma devoid of thylakoids and containing starch grains (Sg). Ol: oil droplets. E. Detail showing mitochondria (Mi) with swelled cristae, chloroplasts (Ch) with apparent thylakoids with plastoglobuli (white arrows) and organelles in degeneration (*). F. Chloroplasts (Ch) with an anomalous format devoid of thylakoids and containing starch grains (Sg) in degeneration. The * indicates organelles undergoing degeneration. G. Detail showing mitochondria (Mt) in the degenerative process within the vacuole (Va).
Bars: A = 20 μm; B = 10 μm; C = 5 μm; D = 2 μm; E = 0.5 μm; F, G = 1 μm.

Table 1 Leaf blade characteristics of *Mentha* x *piperita* L. plants subjected to osmotic stress, including mesophyll thickness, intercellular air spaces, collapsed area thickness, glandular trichome density, palisade cell width, palisade cell length, spongy cell width, and spongy cell length.

Characteristics	PEG 0	PEG 50	PEG 100
Mesophyll thickness (µm)	138.98 ± 6.29 a	139. 85 ± 15.52 a	146.19 ± 6.54 a
Intercellular air space area (μm^2)	256.56 ± 76.14 a	478.97 ± 114.82 ab	592.18 ± 97.95 b
Collapsed area thickness (µm)	-	-	54.75 ± 6.91
Glandular trichome density - adaxial (mm^2)	4.91 ± 1.10 a	3.03 ± 1.40 a	4.12 ± 1.00 a
Glandular trichome density - abaxial (mm^2)	9.32 ± 2.50 a	5.84 ± 2.00 a	6.53 ± 1.70 a
Palisade parenchyma cell width (µm)	18.30 ± 2.64 a	17.33 ± 3.05 a	16.53 ± 2.55 a
Palisade parenchyma cell length (µm)	60.50 ± 8.81 a	53.14 ± 8.68 a	65.88 ± 6.80 a
Spongy parenchyma cell width (µm)	16.91 ± 2.19 a	15.67 ± 5.02 a	15.38 ± 2.96 a
Spongy parenchyma cell length (µm)	29.98 ± 6.09 a	25.16 ± 3.80 a	22.81 ± 2.48 a

Data are means ± SD of eight samples. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test.

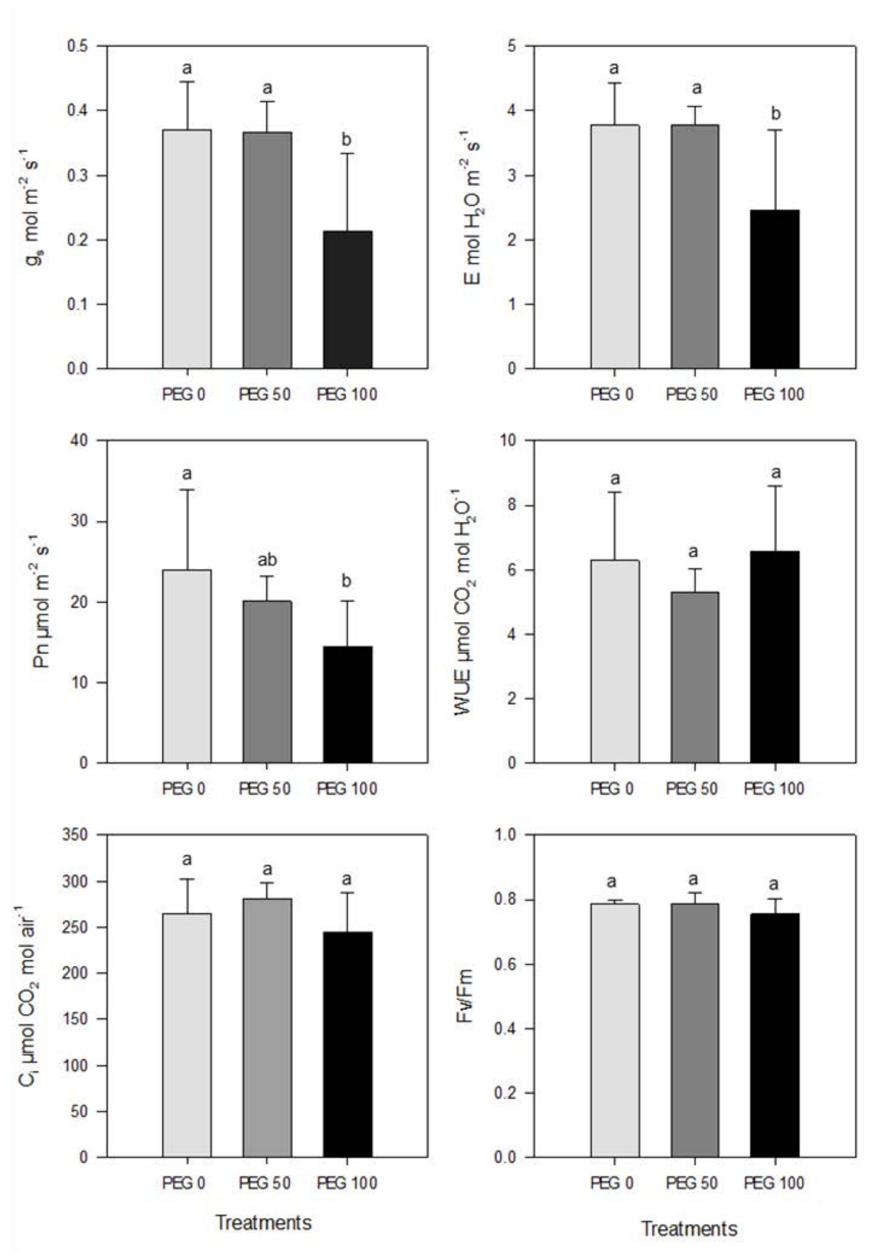

Fig. 7. Effects of osmotic stress on stomatal conductance (g_s ,mol m^{-2} s^{-1}), transpiration (E, mmol H_2O m^{-2} s^{-1}), photosynthetic rate (Pn, µmol m^{-2} s^{-1}), water use efficiency (WUE, µmol CO_2mol H_2O^{-1}), intercellular CO_2 concentration (C_i, µmol CO_2mol air^{-1}) and maximum quantum efficiency of PSII (Fv/Fm) of *Mentha* x *piperita* L. plants subjected to PEG 0, PEG 50 and PEG 100. Data are means ± SD of eight samples. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test.

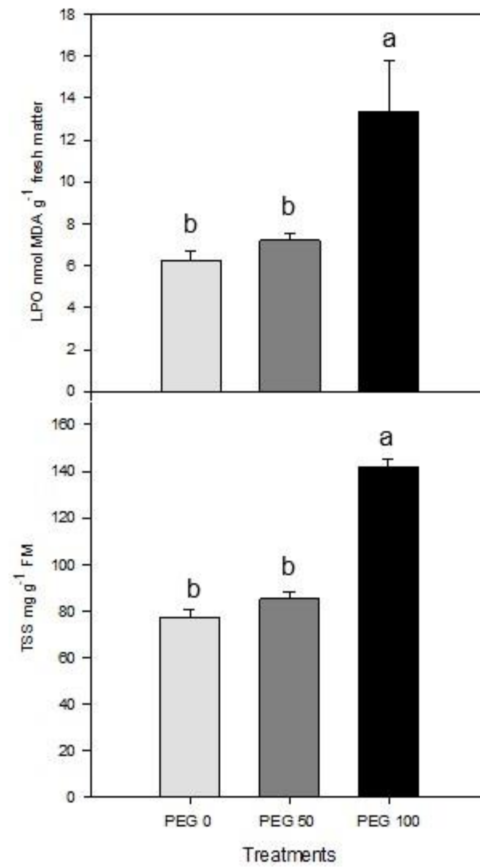

Fig. 8. Effects of osmotic stress on lipoperoxide content (LPO, nmol MDA g^{-1} fresh matter) and total soluble sugars (TSS, mg g^{-1} FM) of *Mentha* x *piperita* L. plants subjected to PEG 0, PEG 50 and PEG 100. Data are means ± SD of eight samples. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test.

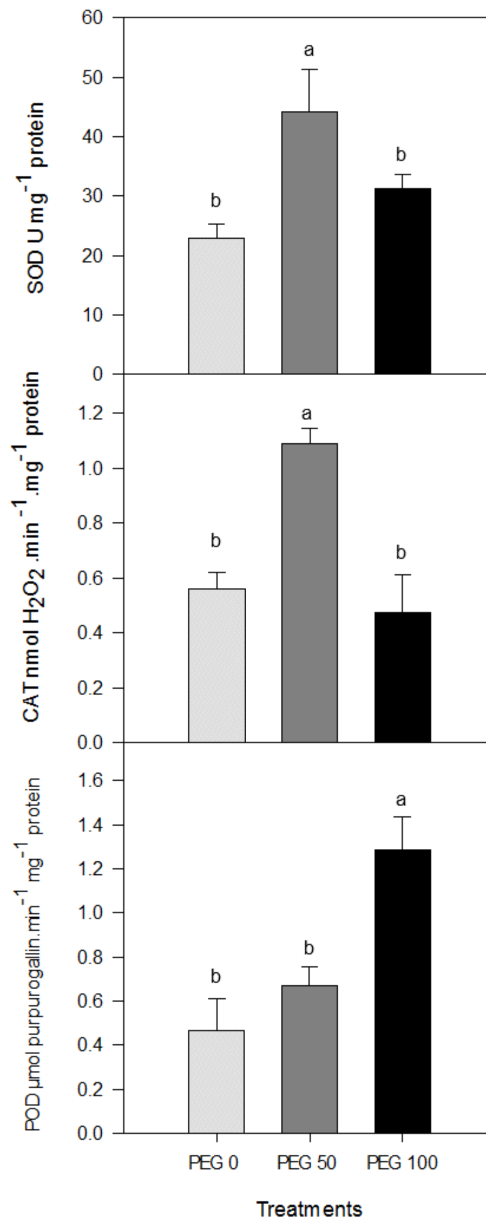

Fig. 9. Effects of osmotic stress on superoxide dismutase (SOD) U mg^{-1} protein, catalase (CAT) nmol consumed H_2O_2.minute^{-1}.mg^{-1} protein and pyrogallol peroxidase (POD) µmol purpurogallin.minute^{-1} mg^{-1} protein of *Mentha* x *piperita* L. plants subjected to PEG 0, PEG 50 and PEG 100. Data are means ± SD of eight samples. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test.

Table 2 Oil content (%) and major essential oil components in percentages of oil, menthone, menthofuran, menthol and pulegone of *Mentha* x *piperita* L. plants subjected to PEG 0, PEG 50 and PEG 100 treatments.

Treatments	Content	Major essential oil components in percentages of oil			
		Menthone	Menthofuran	Menthol	Pulegone
PEG 0	1.32 ± 0.28 a	39.39 ± 2.72 a	32.61 ± 2.90 b	15.34 ± 3.59 a	5.07 ± 0.64 a
PEG 50	1.30 ± 0.46 a	25.76 ± 9.58 b	36.29 ± 3.64 b	15.62 ± 5.72 a	8.08 ± 2.91 a
PEG 100	0.87 ± 0.19 b	18.36 ± 4.92 b	40.83 ± 2.67 a	19.04 ± 2.70 a	8.12 ± 2.95 a
RI[a]		1151	1160	1170	1235
RI[b]		1152	1164	1171	1237

Data are means ± SD of eight samples. Different letters indicate significant differences ($p < 0.05$) according to Tukey's test. RI[a] = retention index calculated; RI[b] = retention index literature.

*Capítulo II – Manuscrito II**

*De acordo com as normas da revista *Industrial Crops and Products*

Organic versus conventional fertilization effects on sweet basil (*Ocimum basilicum* L.) growth in a greenhouse system

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Keywords: nutrients, nitrogen, essential oil, (-)-linalool, eugenol

Abstract

Ocimum basilicum L. (sweet basil) is an essential oil producing crop used in culinary and fragrance applications. The objective of this controlled environment study was to evaluate the effects of organic and conventional fertilization, (applied at two nitrogen rates, 150 and 250 kg N/ha), on plant growth, essential oil yield and chemical profile, and tissue nutrient accumulation in sweet basil. Overall, basil plants fertilized with organic fertilizer at a rate of 150 kg N/ha accumulated greater concentrations of potassium (K) and manganese (Mn). The highest fresh weight was obtained from the plants grown with conventional fertilizer at a rate of 250 kg N/ha. The essential oil yield was the highest in the 250 kg N/ha conventional fertilizer treatment, followed by the yield in the 150 kg N/ha organic fertilizer treatment. Treatments did not affect the oil content (0.34-0.54% range), oil yields (11.4-20.7 mg/pot), and the concentration of eucalyptol, (-)-linalool, bornyl acetate, eugenol, α-trans-bergamotene, germacrene D, ɤ-cadinene and epi-α-cadinol in basil oil. The results from this study demonstrated that organic or conventional fertilizer can alter fresh or dry weight, essential oil yield and nutrient absorption without modifying essential oil composition.

1. Introduction

Ocimum basilicum L. (sweet basil) is a popular food seasoning belonging to the family Lamiaceae which is characterized by a great variability of morphology and chemotypes (Lawrence, 1988; Marotti, 1996). Sweet basil, originally native to India and other regions of Asia, is utilized as an ingredient in Western and Mediterranean diets (Lu et al., 2014). Its leaves contain essential oils of strong aroma. Basil leaves and shoots that are used fresh or dried in culinary applications (Grayer et al., 2004; Ozcan et al., 2005). Some studies have reported that sweet basil contains high concentrations of phenolic compounds (rosmarinic and caffeic acid), which are characterized by high antioxidant capacities (Lee and Scagel, 2009; Surveswaran et al., 2007). Basil extracts are also used in the manufacturing of cosmetic and pharmaceutical products or biopesticides (Keita et al., 2001; Pascual-Villalobos and Ballesta-Acosta, 2003; Umerie et al., 1998).

The high economic value of basil oil is due to the presence of a complex mixture of volatile substances, monoterpenes, sesquiterpenes and their oxygenated analogs present at low concentrations in plants (Lucchesi, 2004). These oil compounds determine the specific aroma and flavors for each basil species and variety. Basil essential oil is synthesized and stored in glandular trichomes of leaves (Sangwan et al., 2001). Generally the main compounds responsible for the typical aroma are 1,8-cineole, methyl cinnamate, methyl chavicol and linalool (Lee et al., 2005). In basil, more than 200 compounds from the essential oil were identified and different chemotypes have been classified for *O. basilicum* according to the essential oil chemical composition (Grayer et al. 1996; Zheljazkov et al., 2008a). The chemical taxonomical range in basil is highly variable. As reported by Lawrence (1988), four chemotypes of basil are (i) methyl chavicol, (ii) linalool, (iii) methyleugenol, (iv) methyl cinnamate in addition to numerous subtypes. Grayer et al. (1996) reported five chemotypes depending on the relative abundance of the following compounds: linalool; methyl chavicol; both linalool and methyl chavicol; both linalool and eugenol; both methyl chavicol and methyl eugenol. In a study with 38 genotypes of sweet basil, Zheljazkov et al. (2008a) identified the following 7 basil chemotypes: On the basis of the oil composition, basil accessions were divided into seven groups: (1) high-linalool chemotype [19–73% (-)-linalool], (2) linalool-eugenol chemotype [six chemotypes with 28–66% (-)-linalool and 5–29% eugenol], (3) methyl chavicol chemotype [six accessions with 20–72% methyl chavicol and no (-)-linalool], (4) methyl chavicol-linalool chemotype [six accessions with 8–29% methyl chavicol and 8–53% (-)-linalool], (5) methyl eugenol–linalool chemotype [two accessions with 37% and 91% methyl eugenol and 60% and 15% (-)-linalool], (6) methyl

cinnamate–linalool chemotype [one accession with 9.7% methyl cinnamate and 31% (-)-linalool], and (7) bergamotene chemotype [one accession with bergamotene as major constituent, 5% eucalyptol, and <1% (-)-linalool].

Basil oil was reported to have antimicrobial, antioxidant (Hussain et al., 2008; Karagözlü et al., 2011), antifungal (Hossain et al., 2014a) and insecticidal (Popovic et al., 2006; Hossain et al., 2014b) properties and some of its components, such as 1,8-cineole, linalool, and camphor, are known to be biologically active (Morris et al., 1979).

There is a strong interest in growing medicinal and aromatic plants in an effort to produce a desired essential oil chemical profile. Active principle constituents from the basil essential oil are affected by diversified factors: water stress (Ekren et al., 2012; Khalid, 2006), salt stress (Barbieri et al., 2012), extraction processes (Yang et al., 2007) and drying methods (Pirbalouti et al., 2013) as well as, cultivation practices as effect of plant nutrition on sweet basil productivity and essential oil composition (Arabaci and Bayram, 2004; Bowes and Zheljazkov, 2004; Golez et al., 2006; Sifola and Barbieri, 2006; Zheljazkov et al., 2008a).

The hypothesis of this study was that organic and conventional fertilizer administered to sweet basil would not result in significant differences in growth, essential oil yield or composition. The objective of this study was to evaluate the effects of organic and conventional fertilizers applied at two nitrogen application rates on growth indicators and essential oil production as well as chemical profile in sweet basil cultivated in a greenhouse system.

2. Material and methods

2.1. Location and Plant material

The study was conducted in the greenhouse of the United States Department of Agriculture, Agricultural Research Service (USDA-ARS), Natural Products Utilization Research Unit in University, Mississippi. Certified organic seeds of sweet basil (*Ocimum basilicum* L.) ‘Genovese’ were obtained commercially from Burpee® (W. Atlee Burpee & Co, Warminster, PA). Seeds were placed in a growth chamber at 25°C and 0012 µMol light for 40 days with Organic Choice Potting Mix (0.10-0.05-0.05) formulated with 55-65% composted bark, sphagnum peat moss pasteurized poultry litter (Miracle Gro® Marysville, OH). After 40 days, basil seedlings (app.15 cm height) were transplanted to 2.0 L pots filled with Organic Choice Potting Mix (Miracle-Gro®, Marysville, OH) and fertilized with conventional NPK fertilizer (10-10-10) (Shake’n Feed Continuous Release Miracle Gro® Marysville, OH) or organic NPK fertilizer (4-3-4) (Organic Fertilizer, Mighty Grow®

Fruitdale, AL). Both the conventional and the organic fertilizers were applied to provide two rates of N; calculated equivalent of 150 and 250 kg N/ha, calculated by the surface area of each pot. Pots were maintained in a greenhouse, with 26 °C day and 25 °C night and watering 8:00 am and 4:00 pm each day for three minutes each time. Plants were harvested on both 02 June (68 days after transplanting) and 03 June (69 days after transplanting) both at full bloom, when the essential oil would be optimal (Topalov, 1962). Basil plants were harvested by cutting at 10 cm above soil surface, the shoot fresh weight was taken. Basil shoots were dried for 3 days and the dried weight was taken.

2.2. Plant tissue analyses

Dry shoot samples were sent to Mississippi State University, Soil Testing and Plant Analysis Laboratory (Starkville, Mississippi) to perform a nutrient analysis to determine the plant tissue levels of N (Nelson and Soommers, 1972), P, K, Ca, Mg, Fe, Zn, B, Mn and Cu (Jones and Steyn, 1973; Isaac and Johnson, 1975).

2.3. Essential oil isolation

Fresh aerial parts (ranging from 69 to 180 g per pot) were subjected to 120 min steam distillation immediately after harvest. Steam distillation was performed using a Clevenger-type apparatus containing n-pentane. Plant material was cut in small pieces and placed into a 2 L round bottom bioflask. The organic phase (n-pentane) was recovered and concentrated to 1.0 mL under a stream of dry nitrogen (Potter, 1996). The essential oil content was calculated by weight as (g) of oil per (g) of fresh herbage (stems, leaves, and flowers) and expressed as (%) of oil in the fresh biomass (corrected for moisture content using the difference between the fresh and dried weight of the biomass samples). The oil yield was expressed in (mg) per pot.

2.4. Gas chromatography

Using a micropipet, 10 μL of oil from each sample was transferred into a 10 mL volumetric flask. Samples were brought to volume with $CHCl_3$. A 1 mL aliquot of each oil sample was placed by glass pipet into a GC vial for analysis.

GC Analysis: A total of eight constituents were identified and quantified in basil essential oil (Fig. 1). Chemical standards and compounds were analyzed on an Agilent 7890 A GC System using GC was equipped with a DB-5 column (30 m x 0.25 mm fused silica cap. column, film thickness of 0.25 μm) operated using the following conditions: injector temp., 240 °C; column temp., 60 to 240 °C at 3 °C/min, held at 240 °C for 5 min; carrier gas, He; injection volume, 5 μl (split on FID, split ratio 25:1); MS mass range from 40 to 650 m/z; filament delay of 3 min; target total ion chromatogram (TIC) of 20,000; a prescan ionization

time of 100 µsec; an ion trap temperature of 150 °C; manifold temperature of 60 °C; and a transfer line temperature of 170 °C; simultaneous detection with MS and FID by splitting the column outlet (1:1). Detector temperature for FID is 300 °C.

GC/MS Analysis: Commercial standards eugenol, obtained from Aldrich (St. Louis, MO), and eucalyptol, (-)-linalool, and (-)-bornyl acetate, all from Fluka (Buchs, Switzerlands), were injected and compared with retention time and mass spectra data of basil essential oil. Compounds α-trans-bergamotene, germacrene D, ɤ-cadinene and epi-α-cadinol were identified by Kovat analysis (Adams, 2009), and comparison of mass spectra with those reported in the NIST mass spectra database. Kovats indices were calculated using equation $KI(x) = 100[(\log RT(x) - \log P_z)/(\log RT(P_{z+1}) - \log RT(P_z)]$ where: $RT(P_z) \leq RT(x) \leq RT(P_{z+1})$ and $P_4 \ldots P_{25}$ are n-paraffins.

GC/FID Analysis: Compounds were quantified by performing area percentage calculations based on the total combined FID area. For example, the area for each reported peak was divided by total integrated area from the FID chromatogram from all reported peaks and multiplied by 100 to arrive at a percent-age. The percentage is a peak area percentage relative to all other constituents integrated in the FID chromatogram.

2.5. Statistical analysis

The effect of fertilizer (2 levels: conventional and organic) and nitrogen rate (2 levels: 150 and 250 kg/ha) on basil growth indicators (fresh weight (g), dry weight (mg), and moisture content (%)), essential oil (percent area (%) of eucalyptol, (-)-linalool, (-)-bornyl acetate, eugenol, α-trans-bergamotene, germacrene D, ɤ-cadinene, epi-α-cadinol, as well as oil yield (mg/pot), and oil content (%), and tissue analysis (N (g/kg), P (g/kg), K (g/kg), Ca (g/kg), Mg (g/kg), Fe (mg/kg), Mn (mg/kg), Zin (mg/kg), Cu (mg/kg), and B (mg/kg)) was determined using a 2 X 2 factorial in 3 blocks design. The two factors of interest (fertilizer, and nitrogen rate) were considered as fixed, and block was considered as random. The analyses was completed using the Mixed Procedure of SAS (SAS Institute Inc. 2010), and further multiple means comparison was completed for significant ($p\text{-value} < 0.05$) and marginally significant ($0.05 < p\text{-value} < 0.1$) effects by comparing the least squares means of the corresponding treatment combinations. Letter groupings were generated using a 5% level of significance. For each response, the validity of model assumptions was verified by examining the residuals as described in Montgomery (2013) and appropriate transformations were applied on responses with violated assumptions. The results reported in the tables and figures are back transformed to the original scale.

3. Results and discussion

3.1 Basil growth indicators

Growth parameters were taken to determine if organic and conventional fertilization and nitrogen rates influence plant development and composition. The results obtained showed that there was an influence by source fertilizer used and N rate. Plant tissue N concentration ranged from 16.4 to 27.5 g/kg (Table 1). Plants under organic fertilizer at 150 kg N/ha accumulated higher concentrations of N, potassium (K) and phosphorus (P). Overall, tissue Ca and Mg were also higher in plants grown at both rates of organic fertilizer (Table 1).

Tissue Mn and Zn were higher in plants grown with organic fertilizer than at the 150 kg N/ha conventional fertilizer treatment (Table 1). Tissue Fe was the highest in plants at the 150 kg N/ha organic fertilizer treatment and the lower in the plants from all other treatments. Tissue B was the highest in plants in the 250 kg N/ha organic fertilizer treatment and lower in the plants from the same conventional treatment, while tissue Cu was not affected by the treatments (Table 1).

These results are in agreement with previous reports. For example, Rasouli-Sadaghian et al. (2010) reported that in sweet basil (*O. basilicum*) fertilized with conventional fertilizers the concentrations were in the following order K, Ca, N, Mg and P, 14.7, 10.5, 9.4, 3.5 and 0.5 mg/pot, respectively, and micronutrients were Fe, 640.1 μg/pot, Cu 67.5 μg/pot, and Mn 304.9 μg/pot. Singh et al. (2014), in trials with sweet basil (*O. basilicum*) cultivated with combined applications of organic manure and inorganic fertilizer in different proportions showed the highest content of N, P and K when plants were fertilized with 50% each of inorganic and organic manures with 3.37% N, 3.14% K and 0.70% P. Based on plants grown with organic manure, the content results were N 2.37%, K 1.63% and P 0.58% better than plants grown with only inorganic fertilizer N 2.28%, K 1.92% and P 0.42%.

Fresh weight yields were higher in the 150 kg N/ha organic and in the 250 kg N/ha conventional fertilizer treatments and lower in the other treatments, however, dry biomass yields were higher in the 250 kg N/ha conventional fertilizer treatment and lower in the other treatments (Table 2). Oil content (0.34-0.54% range), oil yields (11.4-20.7 mg/pot) and moisture content of the biomass (83.7-89.5%) were not affected by the treatments (data not shown). Previous studies with sweet basil found similar essential oil content: 0.39% - 0.97% (Zheljazkov et al., 2008a); 0.07% - 1.37% (Anwar et al., 2005); and 0.04% - 0.70% (Simon et al., 1999). Several studies (Biasi et al., 2009; Silva et al., 2006; Teles et al., 2014) reported the effect of fertilization on essential oil production of medicinal plants shown that such practice does not increase essential oil production, as observed in our study.

Our results are in general agreement with literature reports. According to Carrubba (2009), the response of crops to N fertilization varies according to many factors, as well as environmental conditions, genotype, type of fertilizer and timing of its application. Seufert et al. (2012) reported that when organic systems receive higher quantities of N than conventional systems, organic performance improves, whereas conventional systems don't benefit from more N. Some authors reported that the agronomic efficiency or N uptake in plants decreased with increasing levels of conventional N fertilization. Zheljazkov et al. (2008a) didn't observe increases at fresh herbage yield with increasing N rates in sweet basil plants grown with conventional fertilizer, although when rates of 60, 120 and 180 kg/ha of N were applied, better yields were observed in plants with 60 kg/ha N. Sifola and Barbieri (2006) reported an increase for dry-to-fresh weight ratio in basil plants fertilized with 100 kg ha^{-1} N and also with control treatment (0 N kg ha^{-1}) and less weight was observed at plant with 300 N kg ha^{-1}. Berry et al. (2002) reported N availability as responsible for lower yields in many organic systems, because the release of plant available mineral N from organic sources such as animal manure is slow and this may not provide N at an adequate rate during growing period. Singh et al. (2014) found better results for fresh herb yield in sweet basil when plants were fertilized with 50% each of inorganic and organic manures showing a mean of 20.89 mg/ha, better than only inorganic fertilizer (16.36 mg/ha with 100 kg/ha N rate) or just organic manure (18.85 mg/ha). These authors reported that integration of organic manure with inorganic fertilizer may increase the water holding capacity of soil which helps in dissolution of nutrients resulting in a higher nutrient uptake and better yields.

3.2. Essential oil composition and production of O. basilicum

Eight compounds were identified in the basil oil from this study (Fig. 1) and quantified according to their percent area. Only the concentration of (-)-bornyl acetate (1.6-3.3% of the total oil) was affected by the treatments (Table 3). (-)-Linalool (37.9-46.6%) was the main compound found in the essential oils followed by eugenol (19.6-21.3%), α-trans-bergamotene (11.4-13.3%), epi-α-cadinol (3.3-6.2%), germacrene D (4.0-5.6%), eucalyptol (1.9-5.3%) and ɤ-cadinene (3.0-4.3%) (Table 3). Such a profile is similar to the reports of other authors who evaluated the chemical composition of essential oil from *O. basilicum* plants (Zheljazkov et al. 2008a, 2008b). Examining the oil profile found in our results, sweet basil (*O. basilicum*) cultivar 'Genovese' would be classified as a linalool and eugenol chemotype (Grayer et al. 1996), due to the relative abundance of major compounds in essential oil or to the European basil classified because has linalool with main component (Marotti et al. 1996).

Previous studies reported that linalool and methychavicol as the major compounds found in basil essential oil. This variation may be associated with the origin of the plant, for example, in Australia and India, there was predominance of methychavicol and in France, Seychelles, and the United States was linalool (Lachowicz et al. 1996; Zheljazkov et al. 2008a, 2008b) as we found in our study (Table 3). Moreover, in our study we also didn't find the presence of methychavicol in sweet basil essential oil (Fig. 1).

4. Conclusions

Plants grown under conventional fertilizer responded positively to higher levels of N than organically grown plants, showing both higher fresh and dry weights. On the other hand, basil plants at 150 kg N/ha application rate with organic fertilizer showed better growth parameters, revealing a higher concentration of tissue nutrients and higher fresh and dry weight. Regarding the essential oil composition, the N treatments did not change the oil profile of the basil plants under organic and conventional fertilization. Our results suggest that plants grown with conventional and organic fertilizer showed similar essential oil composition. The chemical profile of basil oil in this study was similar to basil oil in previous reports. This study shows that organic or conventional fertilizer can alter fresh or dry weight and nutrient absorption without modifying essential oil composition. Thus organic fertilizer can be used without compromising essential oil profile; preserving the bioactive compounds and properties that basil has in its essential oil composition.

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Table 1. Macronutrient and micronutrient tissue analysis (g/kg) of *Ocimum basilicum* plants cultivated under organic and mineral fertilization and nitrogen rate.

Fertilizer	Nitrogen (kg/ha)	N (g/kg)	K (g/kg)	Ca (g/kg)	P (g/kg)	Mg (g/kg)	Fe (mg/kg)	Mn (mg/kg)	Zi (mg/kg)	Cu (mg/kg)	B (mg/kg)
Conventional	150	17.00 b	17.03 b	8.03 b	3.17 b	2.73 c	25.70 b	237 b	36.00 c	67.67 a	11.7 ab
	250	17.77 b	13.60 b	10.00 b	3.63 b	3.60 bc	27.33 b	390 ab	69.33 bc	135 a	10.7 b
Organic	150	27.50 a	42.07 a	27.80 a	5.50 a	6.20 ab	50.00 a	673 a	140 ab	129 a	18.0 ab
	250	16.43 b	24.97 b	31.90 a	4.07 ab	6.37 a	29.00 b	609 a	168.3 a	132 a	18.7 a
p-value*											
F		0.02	0.086	0.808	0.049	0.663	0.009	0.29	0.921	0.178	0.721
N		0.034	0.022	0.498	0.387	0.524	0.019	0.65	0.217	0.156	0.943
F*N		**0.052**	**0.002**	**0.003**	**0.015**	**0.006**	**0.005**	**0.013**	**0.004**	0.22	**0.018**

*ANOVA *p*-value for the main and interaction effects of Fertilizer (F), and Nitrogen rate (N). Significant effects that need multiple means comparison are shown in bold. Means followed by the same letter are not significantly different.

Table 2. Growth indicators of *Ocimum basilicum* plants cultivated under organic and mineral fertilization nitrogen rate.

Fertilizer	Nitrogen (kg/ha)	Fresh weight (g)	Dry weight (g)
Conventional	150	29.42 b	4.37 b
	250	55.02 a	8.99 a
Organic	150	46.02 a	4.77 b
	250	33.97 b	3.89 b
p-value*			
F		0.489	0.001
N		0.041	0.023
F*N		**0.001**	**0.001**

*ANOVA *p*-value for the main and interaction effects of Fertilizer (F), and Nitrogen rate (N). Significant effects that need multiple means comparison are shown in bold. Means followed by the same letter are not significantly different.

Table 3. Essential oil composition of *Ocimum basilicum* plants cultivated under organic and mineral fertilization nitrogen rate indentified by retention time and Kovats Index.

	Oil Composition (%)	RI*	KIc.*	KIlit.*	Conventional		Organic		*p*-value
					N (kg/ha)				F*N
					150	250	150	250	
1	eucalyptol	9.091	1036	1031	2.98a	5.27a	2.90a	1.86a	0.127
2	(-)-linalool	11.629	1109	1096	37.91a	46.64a	40.18a	40.79a	0.260
3	(-)-bornyl acetate	19.385	1294	1285	3.28a	2.90a	1.55b	2.67a	**0.080**
4	eugenol	22.372	1361	1359	21.25a	19.58a	19.61a	20.53a	0.590
5	α- trans-bergamotene	25.703	1439	1434	12.98a	11.42a	13.30a	13.77a	0.537
6	germacrene D	27.527	1488	1481	5.55a	3.96a	5.58a	5.12a	0.607
7	ɤ-cadinene	28.83	1521	1513	4.16a	2.98a	4.27a	4.29a	0.534
8	epi-α-cadinol	33.679	1644	1640	5.60a	3.33a	6.22a	5.26a	0.451
	Total identified (%)				93.71	96.07	93.60	94.28	

*RI - retention time observed; KIc. - calculated Kovats Index; KIlit. - Kovats index in literature (Adams, 2007). ANOVA *p*-value for interaction effects of Fertilizer (F) and Nitrogen rate (N). Significant effects that need multiple means comparison are shown in bold. Means followed by the same letter are not significantly different

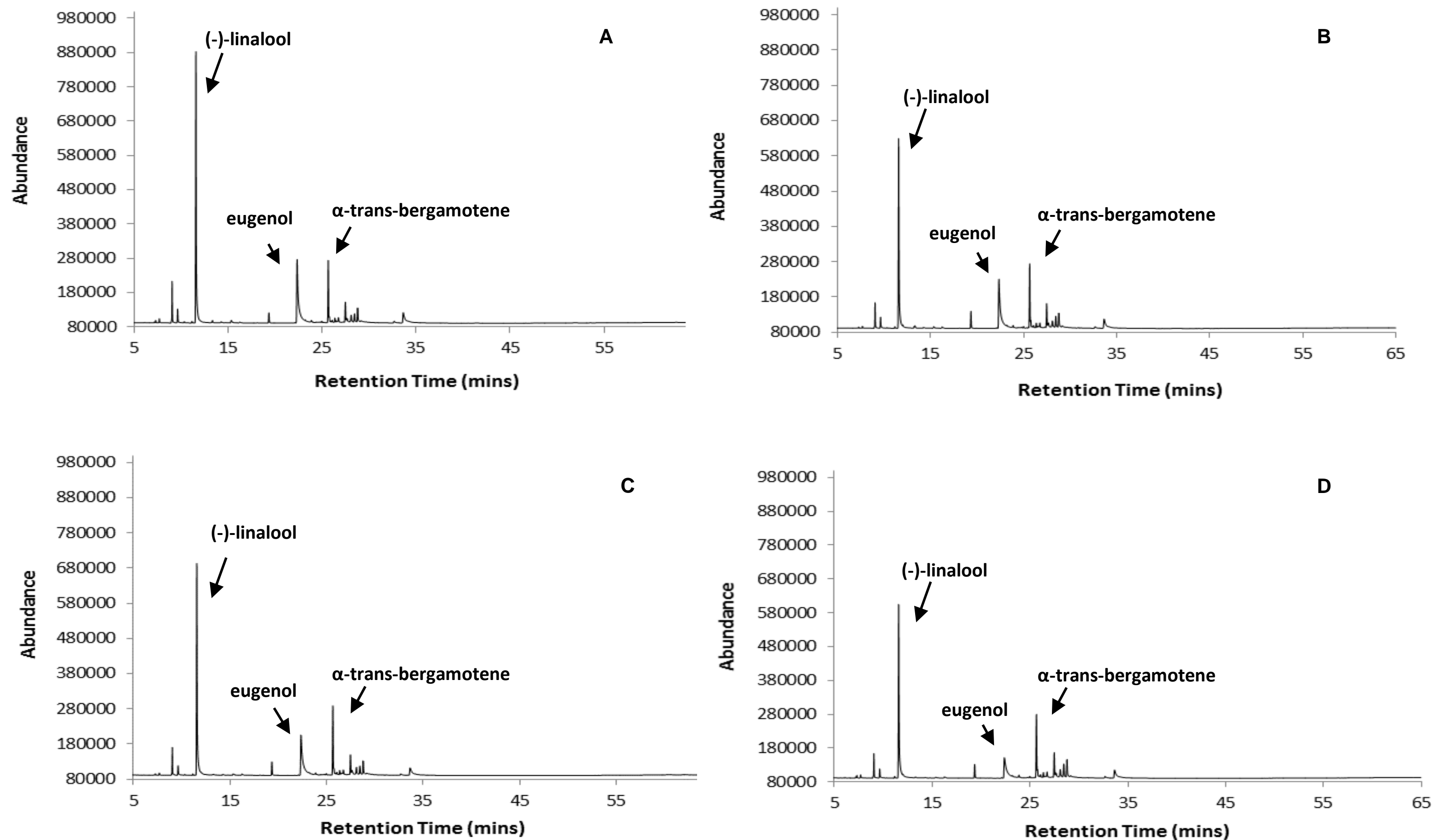

Figure 1. Representative chromatograms (GC-FID) of sweet basil (*Ocimum basilicum*) cultivated under (**A**) organic fertilizer with a rate 150 kg/ha of N, (**B**) conventional fertilizer with a rate 150 kg/ha of N (**C**) organic fertilizer with a rate 250 kg/ha of N and (**D**) conventional fertilizer with a rate 250 kg/ha of N.

*Capítulo III – Manuscrito III**

*De acordo com as normas da revista *Planta Medica*

Antimicrobial and antileishmanial activities of diterpenoids isolated from the roots of *Salvia deserta* Shang

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Abstract

Four diterpenes with biological activity were isolated from *Salvia deserta* roots. Taxodione was considered leishmanicidal, with IC_{50} value of 0.46 µg/mL against *Leishimania donovani* and also exhibited antifungal and antimicrobial activities. Ferruginol displayed the greatest activity (24-h IC_{50} 1.29 mg/L) against the fish pathogenic bacteria *Streptococcus iniae*. The crude extract fraction which contained the isolated compounds 7-*O*-acetylhorminone and horminone showed stronger in vitro antibacterial activity (1.28 µg/mL for *Staphylococcus aureus* and 1.12 µg/mL for methicillin-resistant *S. aureus* - MRSA) than the compounds tested alone. 7-*O*-Acetylhorminone and horminone exhibited a synergistic effect against MRSA (FIC 0.2 µg/mL), and horminone had better activity against *S. aureus* with respect to other compounds isolated from *S. deserta* roots.

Keywords: *Salvia*; Lamiaceae; diterpenoid; leishmania; antimicrobial activity; *Staphylococcus aureus*; *Streptococcus iniae*;

Introduction

The genus *Salvia* is the main genus of the family Lamiaceae with 900 species spread throughout the world [1]. The genus is widely distributed in various regions of the world, including in the Mediterranean area, South Africa, Central and South America, and Southeast Asia [2,3].

Salvia species have been used as antimicrobial [4], antioxidant [5], anticancer [6,7], antiplasmodial [8] and insecticidal [9] agents. One of the most studied species, *Salvia miltiorrhiza* is a well-known herb in traditional Chinese medicine used to treat several diseases and its roots have been reported to contain tanshinone diterpenes [7].

Other secondary metabolites produced by *Salvia* include flavonoids, sequiterpenes, diterpenoids, sesterterpenes and triterpenes [3,10,11]. Diterpenes are the most common secondary metabolites isolated from *Salvia* species [5,12,13] and are found mostly in the roots [14]. However, the literature indicates that some American *Salvia* species also contain diterpenoids in the aerial parts, and in few *Salvia* species, triterpenoids and flavones are present in the roots [15].

Salvia deserta Schang is an herbaceous perennial plant with stems of 35-90 cm with small leaves and purple inflorescences. The leaves and blossoms of *S. deserta* contain a small amount of essential oil (0.02%), which has antimicrobial activity and the seeds contain fatty acids (23%) [16]. In general, its distribution is limited to the mountainous areas of Central Asia, the Caucasus, Western Siberia [17] and in China [12]. The roots of *Salvia* species are used in herbal to treat cough and urethritis [18]. It is well know that different parts of the plant contain different concentrations of active constituents and different phytochemicals [19]. Overall, the chemical composition of *S. deserta* contains organic acids, alkaloids, vitamin C, tannins, flavonoids and quinones [16]. The aerial part of *S. deserta* contains triterpenoids such as ursane, oleanane and lupine derivatives [20] while diterpenes (royleanone, ferruginol, taxodione, etc.), caffeic acid derivatives (rosmarinic acid, lithospermic acid B, etc.) and the steroid daucosterol [12] are found mostly in the roots.

S. deserta has few literature reports on its chemical constituents and no studies have examined the plant's antibacterial and antileishmanial activities. The present study aims to find diterpenoids from this plant with possible biological activity against both human and agricultural pathogens using bioassay-guided fractionation of *S. deserta* roots.

Results and Discussion

A total of 4 diterpenes with biological activity were isolated from *S. deserta* roots: taxodione (**1**), ferruginol (**2**), 7-*O*-acetylhorminone (**3**) and horminone (**4**) (**Fig. 1**). These compounds have previously been identified from *S. deserta* roots by Tezuka [12].

The compounds revealed antibacterial, antifungal and antileishmanial activities. Regarding antimalarial activity, the compounds were found to be very weak, with values of 1.61 – 3.30 µg/mL (data not shown) when compared to artemisinin (IC_{50} < 0.026 µg/mL). No activity was observed for any of the test compounds against *Ae. aegypti* larvae at the highest concentration of 125 mg/L.

Table 1 shows antileishmanial activity of compounds isolated from *S. deserta* roots. The results revealed that only **1** has significant antileishmanial activity with an IC_{50} value of 0.46 µg/mL against *L. donovani*, compared with the standard drug pentamidine (IC_{50} 0.74 µg/mL). Taxodione is a diterpenoid quinone methide (**Fig. 1**), previously isolated from *Plectranthus* and *Taxodium* [21,22,23,24] and is also present in *Salvia* species such as *S. chorassanica* [5], *S. hypargeia* [25], *S. austriaca* [26] and *S. broussonetii* [9]. The antileishmanial activity for **1** isolated from *S. deserta* was consistent with that obtained from the roots of *Clerodendrum eriophyllum* [27] and from the aerial parts of *Plectranthus barbatus* [28].

Among the compounds tested, **2** showed the strongest activity against *Streptococcus iniae*, a bacterium which causes infection in fish species such as tilapia (*Oreochromis* spp.) [29]. This compound is a phenolic abietane diterpene which shows an interesting range of biological activities [5,30] and is common in Asiatic and European *Salvia* species [31]. Based on 24-h IC_{50}, **2** results showed activity at 1.29 mg/L while the other three test compounds showed low or no activities against *S. iniae* at the concentrations used in the study (**Table 2**). The IC_{50} RDCO value of 10.75 was the lowest among the test compounds and also indicates that **2** has strong activity because the relative-to-drug-control oxytetracycline (RDCO) value is closer to "1" and, therefore, more similar in the degree of activity as the drug control oxytetracycline [32]. Previous studies have also isolated bioactive compounds from plants collected in Kazakhstan which showed activity against *S. iniae.* The study by Nakano [33] reported activity against *S. iniae* by emodin and nepodin compounds isolated from *Atraphaxis laetevirens* aerial parts, with 6.4 and 9.1 mg/L 24 h-IC_{50} and 53.2 and 75.5 IC_{50} RDCO values, respectively. Our results suggested that **2** as well as **4** (7.5 mg/L 24 h-IC_{50} and 62.3 IC_{50} RDCO) (**Table 2**) were stronger against *S. iniae* than results found by Nakano [33].

Isolated compounds were also tested in a panel of bacteria and fungi pathogenic to humans. Taxodione had IC_{50} of 0.93 µg/mL and 2.67 µg/mL against *C. neoformans* and *C.* glabrata, respectively (**Table 3**). Taxodione and **2** showed antibacterial activity against *S. aureus* and

methicillin-resistent *S. aureus* (MRSA), with IC_{50} values between 2.78 to 4.00 µg/mL and 2.63 to 2.71 µg/mL, respectively (**Table 4**).

Compound **3** showed weak activity against *S. aureus* and MRSA with IC_{50} of 14.48 µg/mL and 11.11 µg/mL, respectively, while **4** was more potent with IC_{50} of 2.77 and 2.26 µg/mL against *S. aureus* and MRSA, respectively (**Table 5**). Our results are in agreement with those obtained by Ulubelen [4] who reported antibacterial activity for horminone and 7-acetylhorminone against *S. aureus* (MIC of 6.5 and 10 µg/mL, respectively)

Both compounds, are very common in *S. amplexicaulis* [34], *S. eriophora* [35], *S. blepharochlaena* [4] and roots of *S. officinalis* [36]. Ulubelen [37] investigated antibacterial activity from four *Salvia* especies (*S. viridis*, *S. ceratophylla*, *S. caespitosa* and *S. blepharochalasis*) and evaluated antibacterial activity of compounds horminone, 7-acetylhorminone and ferruginol. The authors reported good activitiy for horminone against *S. aureus*, *Staphylococcus epidermis* and *Bacillus subtilis*, and no activity was found for ferruginol.

Fraction C, from which 7-*O*-acetylhorminone and horminone were isolated, showed stronger in vitro antibacterial activity (1.28 µg/mL *S. aureus* and 1.12 µg/mL MRSA, **Table 5**) than the individual compounds alone, and we tested these two compounds in a checkerboard assay to evaluate the effect of each compound in combination with the other on the growth of the test bacteria. According to Jacob and Walker [38], loss of activity from purification is common and may be the result of several factors including instability or synergy of compounds. 7-*O*-Acetylhorminone and horminone were tested in combination at several concentrations for determining the FIC. The compounds demostrated synergistic effect against MRSA with a FIC of 0.2 µg/mL. Thus, the results show that **4** presented better activity against *S. aureus* than other compounds isolated from *S. deserta* roots (**Table 5**).

In conclusion, four compounds belonging to the diterpene class were isolated from the roots of *S. deserta.* This study is the first that has tested crude extract and pure compounds isolated from *S. deserta* roots. The antibacterial activity of these isolated compounds against *S. iniae* is being reported for the first time, and among the four isolated diterpenes, ferruginol appears to be the most promising compound against *S. iniae.* Overall, taxodione exhibited potent antileishmanial, antifungal and antibacterial activity based on the results. However 7-*O*-acetyhorminone and horminone showed synergism when both are combined against MRSA while horminone alone showed better activity against *S. aureus*. This study justifies the use of *S. deserta* roots as a good source of diterpenoids with biological activities and the potential possibilities for the developemnt of novel antipesticidal compounds from this plant.

Materials and Methods

General experimental procedures

A column chromatography (CC): Biotage, Inc. Isolera^{TM} pump (Charlotte, North Carolina) equipped with a Isolera^{TM} flash collector and fixed wavelength (254 nm) detector was utilized.

The HPLC method development work was performed using an Agilent 1200 system equipped with a quaternary pump, autosampler, diode-array detector, and vacuum degasser. Semi prep. HPLC: Column: Zorbax RX-SIL (Agilent) 5 µm 9.4 x 250 nm.

^{13}C-NMR spectra: Bruker 500 MHz (Massachusetts); in $CDCl_3$

GC/MS Analysis: Compounds were analyzed by GC/MS on a Agilent 7890 A GC System. The GC was equipped with a DB-5 column (30 m x 0.25 mm fused silica cap. column, film thickness of 0.25 µm) operated using the following conditions: injector temp., 240 °C; column temp., 60-240 °C at 3 °C/min the held at 240 °C for 5 min; carrier gas, He; injection volume, 1 µL (splitless). The MS mass range from 40 to 650 *m/z*, filament delay of 3 min, target TIC of 20,000, a prescan ionization of 100 µs, an ion-trap temp. of 150 °C, manifold temp. of 60 °C, and a transfer line temp. of 170 °C.

High-Resolution LC/MS Analysis: isolated compounds were prepared in MeOH and injected directly into a 0.3 – mL/min stream of 100% MeOH. Twenty µl of sample (*ca*. 0.1mg/ml) was injected manually at 0.5 min.

Plant Material

The roots of *S. deserta* were collected on June, 22, 2011, while flowering in Almaty, Kazakhstan, at location coordinates: N=43°10'297", E=076°31'788'', H=914 m. A voucher specimen number 7290/25 has been deposited in the Institute of Botany and Phytointroduction herbarium, Almaty, Kazakhstan.

Extraction and Isolation

The 150 g dry roots of *S. deserta* were extracted with 750 mL ethanol, providing 3.6 g of crude extract. Ethanol crude material was extracted again with 700 mL methylene chloride (DCM) providing 2.5 g of crude extract. 1st. Column- Crude extract Fractionation: A portion (1.23 g) of root extract was dissolved in DCM and applied to samplet and adsorbed sample to silica using rotaevaporator. The crude extract was separated by SiO_2 CC on a Biotage Isolera SNAP 100 g column (40-63 µm, 60 Å 39 mm x 15.7 cm) running at 40 mL/min using a hexane/acetone gradient beginning with a linear step gradient from 80% hexane to 20% acetone over 1600 mL followed by 20% hexane to 50% acetone over 800 mL and 50%

hexane to 100% acetone over 400 mL, 100% acetone over 700 mL and finishing with a MeOH wash of 350 mL. Portions of 27 mL fractions were collected and recombined based on thin-layer chromatography (TLC) similarities into eleven fractions (A – L). A total 322.9 mg from Fr. B was fractionated by SiO_2 CC on a Biotage Isolera SNAP 100 g column (40-63 µm, 60 Å 39 mm x 15.7 cm) running at 40 mL/min min using a hexane/diethyl ether gradient beginning with a linear step gradient from 0% hexane to 100% diethyl ether over 2300 mL and finishing with a MeOH wash of 350 mL. Portions of 27 mL fractions were collected and recombined based on TLC similarities into ten fractions (A – J). Then 61 mg of Fr. D was purified in Semi prep. HPLC. The manual injection amount for each sample was 50 µL each time. The method was isocratic 98% hexane: 2% EtOAc for 20 min followed by a 10 min gradient 92% hexane: 8% EtOAc and isocratic 70% hexane: 30% EtOAc for 10 min. For each running, a 5 min column wash with hexane and re-equilibration was performed. Analytes were detected at 254 and 280 nm by diode array detector. Fr. D was purified providing 10.8 mg of taxodione and 9.8 mg of ferruginol.

Taxodione (**1**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 201.04, 181.65, 145.26, 144.93, 139.86, 136.11, 133.94, 125.57, 62.91, 42.83, 42.50, 36.96, 33.23, 32.80, 27.10, 22.09, 21.81, 21.62, 21.21, 18.50. Chemical formula: $C_{20}H_{26}O_3$; Molecular weight: 314.42; the data agree with the published values by Tezuka [12] and Najaran [5].

Ferruginol (**2**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 151.08, 148.26, 131.56, 126.53, 126.33, 110.77, 50.24, 41.54, 38.68, 37.33, 33.28, 33.19, 29.66, 26.53, 24.67, 22.64, 22.45, 21.48, 19.20, 19.12. Chemical formula: $C_{20}H_{30}O$; Molecular weight: 286.45; GC-MS, KI 2327.69, spectra match NIST98 chemical database and literature [39]. The data agree with the published values by Tezuka [12] and Najaran [5].

2nd. Column- Crude extract Fractionation: A portion (958.5 mg) of root extract was dissolved in DCM and adsorbed samplet using rotaevaporator. The crude extract was separated by SiO_2 CC on a Biotage Isolera SNAP 100 g column (40-63 µm, 60 Å 39 mm x 15.7 cm) running at 40 mL/min using a hexane/diethyl ether gradient beginning with a linear step gradient from 0% hexane to 100% diethyl ether over 2300 mL and finishing with a MeOH wash of 350 mL. Portions of 27 mL fractions were collected and recombined based on TLC similarities into nine fractions (A – I). Fr. C provided 51.8 mg and was purified in Semi prep. HPLC. The manual injection amount for each sample was 50 µL each time. The method was gradient 50% hexane: 50% (97% hexane: 3% EtOAc) for 30 min followed by 30 min gradient 50% hexane: 50% (97% hexane: 3% EtOAc). For each running, a 5 min column wash with hexane and re-equilibration was performed. Analytes were detected at 254 and 280 nm by diode

array detector. Fr. C was purified providing 8.8 mg of 7-*O*-acetylhorminone and 11.2 mg of horminone.

7-O-acetylhorminone (**3**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.43, 183.73, 169.42, 150.77, 149.93, 139.45, 124.68, 64.49, 46.12, 40.99, 39.05, 35.79, 32.93, 32.93, 24.62, 24.16, 21.59, 21.05, 19.85, 19.67, 18.79, 18.48. HR-ESI-MS: 375.2220 ($[M + H]^+$, $C_{22}H_{31}O_5^+$; calc. 375.2171). The data agree with the published values by Tezuka [12].

Horminone (**4**). ^{13}C NMR (125 MHz, $CDCl_3$) δ 189.10, 183.87, 151.07, 147.82, 143.17, 124.21, 63.17, 45.73, 41.07, 39.12, 35.78, 33.13, 33.02, 25.78, 23.97, 21.68, 19.86, 19.75, 18.84, 18.37. HR-ESI-MS: 355.1896 ($[M + Na]^+$, $C_{20}H_{28}Na_1O_4^+$; calc. 355.1885). The data agree with the published values by Tezuka [12].

Antimicrobial and antifungal assay

All microorganisms were obtained from the American Type Culture Collection (Manassas, VA) and included the fungi *Candida albicans* ATCC 90028, *C. glabrata* ATCC 90030, *C. krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRSA), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. All microorganisms were tested using modified versions of the CLSI (formerly NCCLS) methods. Drugs controls, ciprofloxacin for bacteria and amphotericin B for fungi, were included in each assay.

Additionally, compounds **3** and **4** were examined for antimicrobial activity using a checkerboard assay, which compares the effect of each test compound alone and in combination and quantitatively assigns activity. The fractional inhibitory concentration (FIC), a ratio of each compound's effect alone and in combination, is calculated to evaluate the extent of synergy or antagonism. The compounds were tested in the same manner as above, but in combination vertically and horizontally on one microplate to ensure every possible combination of test concentration. However, by the nature of the checkerboard assay, there are several choices of concentration combinations used to calculate the FIC. The concentration of **3** or **4** should be high enough that an effect is seen in combination, yet low enough that any inherent activity of **3** or **4** alone is minimal or nonexistent. FIC is calculated using the selected end point (inhibitory concentration that affords 50% growth IC_{50}) using the following formula [38].:

[(IC_{50} (**3**) in combination with (**4**))/IC_{50} (**3**) alone + (IC_{50} (**4**) in combination with (**3**))/IC_{50} (**4**) alone = FIC]

Antimalarial assay

The antimalarial activity was determined against chloroquine sensitive (D6) and chloroquine resistant (W2) strains of *P. falciparum* by measuring plasmodial LDH activity according to the procedure of Makler and Hinrichs [40]. A suspension of red blood cells infected with D6 or W2 strain of *P. falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL Amikacin) was added to the wells of a 96-well microplate containing 10 µL of serially diluted samples (crude extracts, column fractions and pure compounds). Artemisinin and chloroquine were included as the drug controls. IC50 values were computed from the dose response curves generated by plotting percent growth against test concentration.

Antileishmanial assay

Antileshimanial activity was tested in vitro on a culture of *Leishimania donovani* promastigotes employing the almarBlue assay describe by Sodji [41]. Using a 96-well microplate assay, compounds with appropriate dilution were added to the *L. donovani* culture (2 x 10^6 cells/mL). All IC_{50}s and IC_{90}s were calculated using the XLFit fit curve fitting software. The drug controls pentamidine and amphotericin B were used as positive controls.

Antibacterial activity against *Streptococcus iniae*

A culture of *Streptococcus iniae* (isolate LA94-426) was provided by Dr. Ahmed Darwish (formerly at U.S. Department of Agriculture, Agricultural Research Service, Harry K. Dupree Stuttgart National Aquaculture Research Center, Stuttgart, Arkansas). Cultures of *S. iniae* were maintained on plates of Columbia CNA agar containing 5% sheep blood (Remel, Inc., Lenexa, Kansas) to assure purity. Prior to conducting the bioassay, single colonies of the *S. iniae* were used to prepare the assay culture material by aseptically transferring bacterial cells from colonies to 75 mL of 3.8% Mueller-Hinton (MH) broth and then incubating for 18 h at 29±1°C at 150 rpm on a rotary shaker (model C24KC; New Brunswick Scientific, Edison, New Jersey). After incubation, *S. iniae* assay material was prepared by transferring cells from the broth culture to fresh MH broth to form a bacterial cell density of 0.5 McFarland standard. The crude extract from *S. deserta* roots, extract fractions, and isolated pure compounds were evaluated for antibacterial activity using a rapid 96-well microplate bioassay and following the procedures of Schrader and Harries [32]. Oxytetracycline, an antibiotic that can be utilized in medicated feed, was included as a positive drug control. Also, control wells (no test material added) were included in each assay. The initial crude extracts samples were dissolved in dichloromethane. Extract fractions and isolated pure compounds obtained later were also dissolved separately in dichloromethane while the drug control was dissolved in ethanol. Final test concentrations of the crude extracts and extract

fractions in the microplate wells were 0.1, 1.0, 10.0, and 100.0 mg/L. Final concentrations of test compounds and drug controls were 0.01, 0.1, 1.0, 10.0, and 100.0 μM. The assays were realized in triplicate. In order to determine the 24-h 50% inhibition concentration (IC_{50}) and minimum inhibition concentration (MIC), sterile quartz 96-well microplates (Hellma Cells, Inc., Forest Hills, New York) were used. Initially, dissolved test material or drug control were micropippeted separately into individual microplate wells (10 μL/well), and solvent was allowed to completely evaporate before 0.5 MacFarland bacterial culture (see Schrader and Harries, [32]) was added to the microplate wells (200 μL/well). Microplates were incubated at 29±1°C (VWR model 2005 incubator; Sheldon Manufacturing, Inc., Cornelius, Oregon). A Packard model Spectra Count microplate photometer (Packard Instrument Company, Meriden, Connecticut) was used to measure the absorbance (630 nm) of the microplate wells at time 0 and 24 h. The means and standard deviations of absorbance measurements were calculated, and compared to controls to determine the 24-h IC_{50} and MIC for each crude extract, extract fraction, and isolated test compound (see Schrader and Harries, [32]). The 24-h IC_{50} and MIC results for each test fraction and compound were divided by the respective 24-h IC_{50} and MIC results obtained for the positive control oxytetracycline to determine the relative-to-drug-control oxytetracycline (RDCO) values.

Mosquito Larval Bioassay

Larvae of *Aedes aegypti* L. used in these screening bioassays were hatched from the eggs obtained from a laboratory colony maintained at the Mosquito and Fly Research Unit at the Center for Medical, Agricultural and Veterinary Entomology, USDA-ARS, Gainesville, Florida. The eggs were hatched and the larvae were maintained at a temperature of 27 ± 2˚C and 60 ± 10% RH in a photoperiod regimen of 12:12 h (L:D). Bioassays were conducted using the system described by Ali [42] to determine the larvicidal activity of root extract of *S. deserta* extract and pure compounds against *Ae. aegypti*. Five 1-d-old larvae were transferred to individual wells of a 24-well tissue culture plates in a 30-40 μL droplet of water. Fifty microliters of larval diet of 2% slurry of 3:2 beef liver powder (Now Foods, Bloomingdale, Illinois) and Brewer's yeast (Lewis Laboratories Ltd., Westport, CT) and 1 mL of deionized water were added to each well by using a Finnpipette stepper (Thermo Fisher, Vantaa, Finland). All the extracts to be tested were diluted in DMSO. Eleven microliters of the test chemical was added to the labeled wells, while 11 μL of DMSO was added to the control treatments. After treatment application, the plates were swirled in clock-wise and counter-clockwise motions and from front to back and side to side five times to ensure even mixing of the treatments. Permethrin (46.1% *ci*s – 53.2% *trans*, Chemical Service, West Chester, PA) at

0.025 mg/L was used as positive control. Larval mortality was recorded at 24-h post treatment.

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Conflicts of Interest

The authors declare no conflict of interest.

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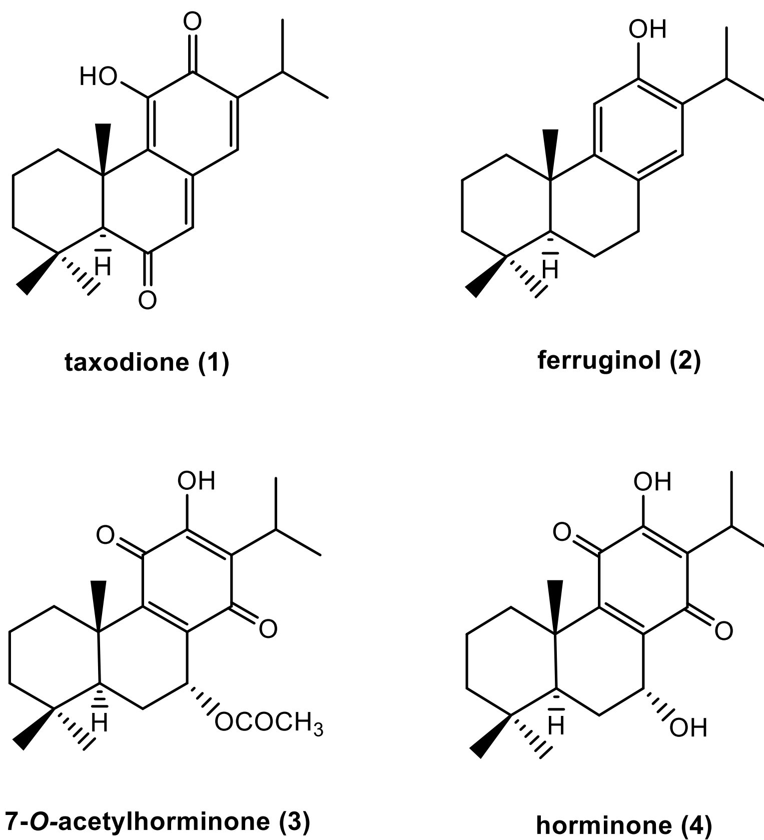

Fig. 1 Structures of taxodione (**1**), ferruginol (**2**), 7-*O*-acetylhorminone (**3**) and horminone (**4**) from *S. deserta* roots.

Table 1 Antileishmanial activity of diterpenoids of *S. deserta* roots.

Compound	***L. donovani***	
	IC_{50} (μg/mL)	***IC_{90} (μg/mL)***
Amphotericin B	0.15 ± 0.02	0.25 ± 0.03
Pentamidine	0.74 ± 0.08	1.83 ± 0.05
taxodione (**1**)	0.46 ± 0.16	0.71 ± 0.34
ferruginol (**2**)	3.26 ± 0.30	6.72 ± 0.56
7-*O*-acetylhorminone (**3**)	7.37 ± 0.30	10.27 ± 0.66
horminone (**4**)	9.78 ± 1.01	20.67 ± 0.88

IC_{50} = is the concentration that affords 50% inhibition of growth; IC_{90} = is the concentration that affords 90% inhibition of growth; as µg/mL and are the mean ± S.D.

Table 2 Antibacterial activity of diterpenoids from *S. deserta* roots against *Streptococcus iniae*.

Compound	***24-h IC_{50}*** [a]	***MIC*** [b]	***IC_{50} RDCO***	***MIC RDCO***
oxytetracycline HCl	0.12 ± 0.03	0.05 ± 0		
taxodione (**1**)	13.7 ± 0.8	31.4 ± 0	144.1 ± 36.7	628.8 ± 0
ferruginol (**2**)	1.29 ± 0.14	2.86 ± 0	10.75 ± 1.2	57.2 ± 0
oxytetracycline HCl	0.12 ± 0.03	0.50 ± 0		
7-O-acetylhorminone (**3**)	17.8 ± 6.6	20.6 ± 16.8	148.0 ± 54.5	411.4 ± 336.6
horminone (**4**)	7.5 ± 2.5	18.3 ± 14.9	62.3 ± 20.8	365.2 ± 298.8

[a]24-h IC50 = 50% inhibition concentration in mg/L and are the mean ± S.E. [b]MIC = Minimum inhibition concentration in mg/L and are the mean ± S.E. RDCO = Relative-to-drug-control oxytetracycline; numbers closer to “1” indicate stronger activity.

Table 3 Antifungal activities of diterpenoids *S. deserta* roots.

Compound	*C. glabrata*			*C. krusei*			*C. neoformans*			*A. fumigatus*			*C. albicans*			*Test Conc.(μg/mL)*
	Concentration (μg/mL)															
	IC_{50}	*MIC*	*MFC*	IC_{50}	*MIC*	*MFC*	IC_{50}	*MIC*	*MFC*	IC_{50}	*MIC*	*MFC*	IC_{50}	*MIC*	*MFC*	
amphotericin B	0.13	0.63	0.63	0.26	0.63	1.25	0.19	0.63	1.25	0.60	1.25	2.5	0.10	1.25	1.25	5-0.005
taxodione (**1**)	2.67	5.00	5.00	5.39	10.00	20.00	0.93	5.00	5.00	16.17	-	-	11.69	20.00	-	20-0.02
ferruginol (**2**)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20-0.02

(-) not active higher concentration 20 μg/mL; IC_{50} 50% growth inhibition; MIC, minimum inhibitory concentration (lowest concentration that allows no detectable growth); MFC, minimum fungicidal concentration (the lowest concentration that kills the fungus).

Table 4 Antimicrobial activities of diterpenoids of *S. deserta* roots.

Compound	***S. aureus***			***MRSA***			***E. coli***			***P. aerugiosa***			***M. intracellulare***			***Test Conc. (µg/mL)***
	Concentration (µg/mL)															
	IC_{50}	***MIC***	***MBC***	***IC_{50}***	***MIC***	***MBC***	***IC_{50}***	***MIC***	***MBC***	***IC_{50}***	***MIC***	***MBC***	***IC_{50}***	***MIC***	***MBC***	
ciprofloxacin	0.11	0.25	0.50	0.12	0.25	1.00	0.01	0.03	0.06	0.09	0.50	1.00	0.47	1.00	1.00	1-0.001
taxodione (**1**)	2.78	10.00	20.00	2.63	10.00	10.00	-	-	-	-	-	-	-	-	-	20-0.02
ferruginol (**2**)	4.00	10.00	-	2.71	5.00	-	-	-	-	-	-	-	-	-	-	20-0.02

(-) not active higher concentration 20 µg/mL; IC_{50} 50% growth inhibition; MIC, minimum inhibitory concentration (lowest concentration that allows no detectable growth); MBC, minimum bactericidal concentration (the lowest concentration that kills the bacteria).

Table 5 Antimicrobial activities and Fractional inhibitory concentration (FIC) of compounds 7-*O*-acetylhorminone and horminone against *S. aureus* and MRS.

Compound	***S. aureus***			***MRSA***			***Test Conc. (µg/mL)***
	Concentration (µg/mL)						
	IC_{50}	***MIC***	***MBC***	***IC_{50}***	***MIC***	***MBC***	
ciprofloxacin	0.08	0.5	0.5	0.08	0.5	0.5	1-0.001
7-*O*-acetylhorminone (**3**)	14.48	-	-	11.11	-	-	20-0.02
horminone (**4**)	2.77	-	-	2.26	-	-	20-0.02
fraction C [app. 45% (**3**) + 55%(**4**)]	1.28	-	-	1.12	-	-	20-0.02

Fractional Inhibitory Concentration (FIC)

IC_{50} (3)	**In combination with µg/mL (4)**	**IC_{50} (4)**	**In combination with µg/mL (3)**	***FIC****
S. aureus				
6.35	0.63	0.53	1.25	0.6
MRSA				
0.53	0.63	0.41	0.63	0.2

*FIC: 0.5 ≤ synergistic; 0.51–1 = additive; 1.1–2 = indifferent; >2 = antagonistic. (-) not active higher concentration 20 µg/mL; IC_{50} 50% growth inhibition; MIC, minimum inhibitory concentration (lowest concentration that allows no detectable growth); MBC, minimum bactericidal concentration (the lowest concentration that kills the bacteria).

Considerações Finais

Considerações finais

Este estudo teve por objetivo geral a investigação de metabólitos secundários de três espécies da família Lamiaceae, *M.* x *piperita*, *Ocimum basilicum* e *Salvia deserta,* submetidas ou não à variação de fatores abióticos como água e nutrição. Nos capítulos I e II a abordagem foi o óleo essencial e respostas fisiológicas das espécies *M.* x *piperita* e *Ocimum basilicum* cultivadas em diferentes condições com variação de fatores abióticos. No capítulo III o enfoque foi à identificação de compostos bioativos presentes em raízes de *Salvia deserta.*

O estudo sobre os efeitos do estresse osmótico na *M.* x *piperita,* permitiu observar que as plantas submetidas à maior dose, de PEG e igual a 100 g L^{-1}, reduziram o potencial água das folhas, diminuíram as trocas gasosas, aumentaram o conteúdo de açúcares totais e ativaram o sistema de defesa antioxidante, que não se revelaram suficientes para conter a degradação das membranas, pois danos ultraestruturais foram observados e houve diminuição do conteúdo e qualidade do óleo essencial.

O estudo com manjericão doce comparou os efeitos da fertilização orgânica e convencional em plantas cultivadas em casa de vegetação. As plantas cultivadas com fertilizante convencional, dose 250 kg ha^{-1} N, mostraram maior massa fresca e seca do que plantas cultivadas com fertilizante orgânico. Os tratamentos não afetaram o conteúdo, produção e composição do óleo essencial, que apresentou composição química semelhante a outros trabalhos presentes na literatura. Assim, o fertilizante orgânico pode ser utilizado sem alterar o perfil do óleo essencial de manjericão, uma vez que, todos os compostos bioativos estavam presentes na avaliação de sua composição.

A bioprospecção de metabólitos bioativos em extratos de raízes de *S. deserta* avaliou o potencial antimicrobiano e antileishmanicida de diterpenos. O composto *taxodione* apresentou atividade antileishmanicida, antifúngica e antimicrobriana. *Ferruginol* apresentou atividade contra bactéria presente em peixes, *Streptococcus iniae.* A fração do extracto bruto, a qual continha os compostos isolados *7-O-acetylhorminone* e *horminone* mostraram forte atividade antibacteriana para o *Staphylococcus aureus* e *S. Aureus resistente á meticilina* (MRSA) do que os compostos testados isoladamente. Os compostos *7-O-Acetylhorminone* e *horminone* exibiram efeito sinérgico contra o MRSA, e *horminone* apresentou melhor atividade contra *S. Aureus*. O presente trabalho contribuiu para o conhecimento do cultivo das espécies medicinais estudadas visando avaliar a produção e qualidade de seus óleos essenciais e quanto à bioprospecção de produtos naturais com atividades biológicas.