Ethylene synthesis inhibition effects on oxidative stress and *in vitro* conservation of *Lippia filifolia* (Verbenaceae)

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Abstract

This study aimed to investigate the effects of ethylene biosynthesis inhibitors on oxidative metabolisms and the *in vitro* conservation of *Lippia filifolia*, using the lipid peroxidation index (TBARS), antioxidative enzymes and pigments as biomarkers. We found that EDTA, sodium thiosulfate (STS) and especially Co had protective effects on oxidative stress in tissues cultured *in vitro*, resulting in a delay of the senescence and the reduction of subcultures frequency, contributing to the germplasm conservation of this species.

Keywords: ethylene inhibitors, TBARS, ROS, micropropagation, germplasm conservation.

Efeitos da Inibição da síntese do etileno no estresse oxidativo e na conservação *in vitro* de *Lippia filifolia* (Verbenaceae)

Resumo

O objetivo deste estudo foi investigar os efeitos de inibidores da biossíntese do etileno no metabolismo oxidativo e na conservação *in vitro* de *Lippia filifolia*. Para isso, foram avaliados o índice de peroxidação lipídica (TBARS), a atividade de enzimas antioxidativas e o conteúdo de pigmentos fotossintéticos e de antocianinas. Os resultados evidenciaram que o EDTA, o tiossulfato de sódio (STS) e, especialmente, o Co apresentaram ação protetora sobre o estresse oxidativo nos tecidos, o que resultou em atraso no início da senescência das culturas e na redução da frequência dos subcultivos, contribuindo para a conservação do germoplasma dessa espécie.

Palavras-chave: inibidores de etileno, TBARS, EROs, micropropagação, conservação de germoplasma.

1. Introduction

One of the main diversity centres of the genus *Lippia* is located in Cadeia do Espinhaço, Minas Gerais state, Brazil. In this environment, some endemic species have been threatened by severe destruction, particularly caused by mining activities (Giulietti et al., 1987). Micropropagation offers an alternative method to conventional vegetative propagation and germplasm conservation, especially for endemic and endangered species. Plant tissue culture involves manipulations, and the explants respond to environmental, physiological and metabolic changes. As a consequence, there is an increase in the demand for antioxidant protection to compensate for the pro-oxidative changes that occur in parallel to metabolic and developmental transitions (Benson, 2000).

In vitro propagation has limitations, especially when the accumulation of ethylene in culture vessels is severe and/or the genotypes exhibit sensibility to this phytohormone (Ievinsh et al., 2000). The ethylene biosynthesis is relatively simple and different substances interfere in specific points of the pathway (Wang et al., 2002). Cobalt (Co) ions, chelating agents (EDTA, EGTA) and salicylic acid (SA) prevent or reduce aminocyclopropane carboxylic acid (ACC) conversion to ethylene (Wang et al., 2002). Silver nitrate ($AgNO_3$) and silver thiosulfate (STS) also affect ethylene activity, reducing sensitivity and also the negative effects on plant tissues (Wang et al., 2002).

The ethylene effects on *in vitro* morphogenesis are not fully understood, but the role of this hormone in senescence has been widely reported (Roustan et al., 1989; Kumar et al., 1998; Wang et al., 2002). Senescence is a natural phenomenon related both to ethylene and oxidative stress (Wang et al., 2002). The reactive oxygen species (ROS) are toxic molecules naturally produced as a result of aerobic metabolism, and therefore they should be rapid and efficiently scavenging, which occurs due to different antioxidant systems (Scandalios, 1993; Anderson et al., 1995; Mittler, 2002).

The aim of this work was to investigate the effects of ethylene biosynthesis inhibitors on oxidative stress and the *in vitro* conservation of *Lippia filifolia*, using the lipid peroxidation index (TBARS), antioxidative enzymes activity and the accumulation of pigments as biomarkers.

2. Materials and Methods

2.1. Tissue culture

Apical shoots (average 10 mm) of *Lippia filifolia* Mart. and Schauer ex Schauer previously established in MS media (Murashige and Skoog, 1962) without growth regulators were used. The explants were inoculated on MS medium supplemented with 10 nM of α-naphthaleneacetic acid (NAA). Additionally, different substance inhibitors of synthesis or ethylene action were added: $AgNO_3$ (6, 12 or 18 mM), SA (80, 160 or 240 mM), Co ($CoCl_2.6H_2O$ - 20, 40 or 60 mM), EDTA (45, 90 or 135 mM) or STS (6, 12 or 18 mM). The pH was adjusted to 5.7 ± 0.1 before autoclaving. Fifteen millilitres of culture medium were added into test tubes (2.5 x 15 cm). The tubes were capped with polypropylene closures and further sealed with a 9 μm PVC film. Cultures were maintained for 60 days in a growth chamber at 16 h photoperiod, 26/20 °C (day/night temperature), and an irradiance of around 36 μmol m^{-2} s^{-1}.

2.2. TBARS evaluation

Thiobarbituric acid reactive substances (TBARS) in tissues were evaluated as described by Cakmak and Horst (1991). Tissues were homogenised in 4 mL of 1% (m/v) trichloroacetic acid (TCA). After purification, 1 mL of supernatant was added to 3 mL of 0.5% (m/v) thiobarbituric acid (TBA) in 20% (m/v) TCA. The test tubes were capped and incubated in a water bath at 95 °C, for 2 h. The reaction was stopped by cooling the test tubes in an ice bath. After clarification by centrifugation, supernatant absorbance was evaluated at 532 and 660 nm. The MDA-TBA (TBARS) complex formation was estimated using a molar extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1968).

2.3. Enzymatic analysis

Enzymatic extracts to determine activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), peroxidase (POD, EC 1.11.1.7) and polyphenoloxidase (PPO, EC 1.10.3.2, EC 1.10.3.1, EC 1.14.18.1) were obtained by tissue homogenisation in 0.1 M potassium phosphate buffer, pH 6.8 with 0.1 mM EDTA. The homogenates were filtered and centrifuged, and the supernatants were used to perform the enzyme assays. SOD activity was measured by adding aliquots of the enzymatic extracts to the reaction mixture containing 13 mM L-methionine, 75 mM *p*-nitroblue tetrazolium (NBT), 100 nM EDTA and 2 μM riboflavin in 50 mM sodium phosphate buffer, pH 7.8 (Del Longo et al., 1993). The enzyme catalysis was carried out in a chamber illuminated by a 15-W fluorescent lamp for 3 min (Giannopolitis and Ries, 1977). Photoreduction of NBT to blue formazan was measured by the increase of absorbance to 560 nm. One unit of SOD is defined as the amount of enzyme necessary to inhibit the NBT photoreduction by 50% (Beauchamp and Fridovich, 1971). POD activity was assayed by adding aliquots of the enzymatic extracts to 5 mL of a reaction mixture containing 25 mM potassium phosphate buffer, pH 6.8, 20 mM pyrogallol and 20 mM H_2O_2 (Kar and Mishra, 1976). After 1 min, the reaction was stopped by adding 0.5 mL of H_2SO_4 5% (v/v). Absorbance was evaluated at 420 nm. POD activity was measured by using a molar extinction coefficient of 2.47 mM^{-1} cm^{-1} (Chance and Maehley, 1955). PPO activity was measured as described for POD (Kar and Mishra, 1976), except for the exclusion of H_2O_2 from the incubation media. CAT activity was measured by adding aliquots of enzyme extract to 3 mL of a mixture containing 12.5 mM H_2O_2 in 50 mM of potassium phosphate buffer, pH 7.0 (Havir and McHale, 1987). The enzyme activity was measured as the absorbance decreased to 240 nm, assuming a molar extinction coefficient of 36 mM^{-1} cm^{-1} (Anderson et al., 1995).

2.4. Pigment analysis

For the chlorophylls and carotenoids analysis, samples were extracted from 5.0 mL of acetone 80% (v/v). Chlorophylls *a* and *b*, total chlorophyll and carotenoids were determined according to Lichtenthaler (1987). Total anthocyanins were measured following the procedure described by Mancinelli (1990), using 5.0 mL of methanol-HCl (99:1, v/v). All extracts were clarified by centrifugation before spectrophotometric determinations.

2.5. Statistical analysis

The experiment was carried out in a completely randomised design. The ANOVA and the Scott-Knott test were performed with 5% probability, using the SAEG software 9.1.

3. Results and Discussion

3.1. Explants regeneration and senescence

The regeneration rate of the explants was 100%, regardless of the treatment. Explants which were maintained in a culture medium supplemented with EDTA or STS and, especially, with Co showed 92-95% of rooted microcuttings. Under these conditions, the senescence

rate was 50% slower (150 days) than in explants cultured in medium with SA and $AgNO_3$ (100 days), which was reinforced by a marked chlorosis and faster leaf abscission.

3.2. Lipids peroxidation (TBARS index)

In this study, it was possible to observe significant reductions in the TBARS index at the highest level of SA, in the presence of EDTA and STS, regardless of the concentration, and especially when Co was added to the culture medium (Table 1). On the other hand, lipid peroxidation was stimulated as the $AgNO_3$ increased in the culture media. This stimuli is attributed to increased levels of ethylene in response to silver ions (Molassiotis et al., 2005), which may be caused by its toxicity or by the reduction of tissue sensitivity to ethylene. This fact can be metabolically interpreted as a deficiency of this hormone causing an increase in the biosynthesis (Theologis, 1992).

3.3. Enzymes activities

Several enzymes are related to the protection of protoplasm and cell integrity against oxidative stresses (Anderson et al., 1995). We observed that SOD activity was reduced when Co and EDTA were added, regardless of their concentration, as well as STS at the highest level (Table 1). SOD activity is usually associated with POD or CAT activities, enzymes that use H_2O_2 produced in the reaction performed by SODs (Cakmak and Horst, 1991; Arora et al., 2002; Mittler, 2002). The combined action of SODs, CATs and PODs, associated to low-molecular-weight antioxidant substances, can effectively eliminate, scavenge and/or immobilise toxic oxygen species (Scandalios, 1993; Siegel, 1993).

POD activities were higher when comparing the control, especially in treatments with $AgNO_3$ and SA (Table 1). In contrast, the enzyme activity was lower in the presence of Co. The PODs action on electron donor molecules, using H_2O_2 as substrate, has an important contribution on ROS detoxification (Gaspar et al., 1985). PODs can also degrade indole-3-acetic acid (IAA) and the aminocyclopropane carboxylic acid (ACC), which are important intermediaries in the ethylene biosynthesis pathway (Wang et al., 2002). ACC oxidases are PODs associated with membranes that can regulate ethylene production (Gaspar et al., 1985; Wang et al., 2002). Gaspar et al. (1985) suggested that ethylene also regulates the activities of phenylalanine ammonia-lyases (PALs) and acid PODs, enzymes related to lignification. Sakamoto et al. (2008) observed that H_2O_2 is involved in abscission signalling, and also that ethephon-induced abscission is suppressed by inhibitors of H_2O_2 production, suggesting

Table 1 - TBAR [mmol g^{-1}(f.m.)] contents, SOD [10^{-3} U g^{-1} (f.m.)], POD [mmol g^{-1} (f.m.) min^{-1}], PPO [mmol g^{-1} (f.m.) min^{-1}] and CAT [mmol g^{-1} (f.m.) min^{-1}] activities, chlorophyll *a*+*b* [mg g^{-1} (f.m.)], carotenoids [mg g^{-1} (f.m.)] and anthocyanin [A_{530} g^{-1} (f.m.)] contents and chlorophyll a/b ratio in *in vitro* cultured *Lippia filifolia*. Means followed by the same letter (for each substance) are not significantly different by the Scott-Knott test at 5% probability. [(+) higher than the control; (-) smaller than the control; (=) equal to the control)]. (*n = 5* replications)

Treatment	TBAR	SOD	POD	PPO	CAT	Chl *a* + *b*	Carot.	Chl*a* / Chl*b*	Anthoc.
Control	0.607	0.499	0.157	0.030	1.436	0.573	0.185	2.981	3.740
$AgNO_3$ 6 µM	1.003 c^{+}	0.721 $b^{=}$	0.209 $b^{=}$	0.071 c^{+}	1.792 $b^{=}$	0.303 a^{-}	0.212 $a^{=}$	0.302 b^{-}	0.995 b^{-}
$AgNO_3$ 12 µM	1.906 b^{+}	1.459 a^{+}	0.534 a^{+}	0.205 a^{+}	4.514 a^{+}	0.230 b^{-}	0.153 b-	0.352 b^{-}	1.800 a^{-}
$AgNO_3$ 18 µM	2.931 a^{+}	0.573 $b^{=}$	0.554 a^{+}	0.159 b^{+}	3.720 a^{+}	0.207 b^{-}	0.127 c^{-}	0.580 a^{-}	1.656 a^{-}
SA 80 µM	1.005 a^{+}	0.505 $a^{=}$	0.246 c^{+}	0.087 c^{+}	4.836 a^{+}	0.235 b^{-}	0.216 a^{+}	0.285 b^{-}	4.020 $a^{=}$
SA 160 µM	0.672 $a^{=}$	0.793 a^{+}	0.324 a^{+}	0.127 a^{+}	3.504 b^{+}	0.267 a^{-}	0.220 a^{+}	0.427a^{-}	2.933 b^{-}
SA 240 µM	0.476 b^{-}	0.695 $a^{=}$	0.272 b^{+}	0.108 b^{+}	3.618 b^{+}	0.283 a^{-}	0.209 a^{+}	0.515a^{-}	3.454 b^{-}
Co 20 µM	0.116 a^{-}	0.266 b^{-}	0.082 b^{-}	0.020 c^{-}	1.091 b^{-}	0.138 a^{-}	0.105 a^{-}	1.625 b^{-}	2.025 a^{-}
Co 40 µM	0.070 a^{-}	0.403 a^{-}	0.163 $a^{=}$	0.045 a^{+}	1.432 $a^{=}$	0.092 b^{-}	0.055 b^{-}	1.377 b^{-}	2.480 a^{-}
Co 60 µM	0.072 a^{-}	0.241b^{-}	0.128 $a^{=}$	0.034 $b^{=}$	1.204 b^{-}	0.035 c^{-}	0.033 b^{-}	3.121 $a^{=}$	0.529 b^{-}
EDTA 45 µM	0.313 a^{-}	0.519 $a^{=}$	0.237 a^{+}	0.055 a^{+}	2.410 a^{+}	0.082 b^{-}	0.052 a^{-}	3.473 a^{+}	2.535 b^{-}
EDTA 90 µM	0.165 b^{-}	0.317 b^{-}	0.200 b^{+}	0.041 b^{+}	1.672 b^{+}	0.095 a^{-}	0.039 b^{-}	1.555 b^{-}	5.013 a^{+}
EDTA 135 µM	0.152 b^{-}	0.370 $a^{=}$	0.143 $c^{=}$	0.037 $b^{=}$	1.718 b^{+}	0.085 b^{-}	0.035 b^{-}	1.939 b^{-}	3.929 $a^{=}$
STS 6 µM	0.286 a^{-}	0.973 a^{+}	0.268 a^{+}	0.068 a^{+}	2.300 a^{+}	0.173 a^{-}	0.042 b^{-}	1.912 c^{-}	2.621 b^{-}
STS 12 µM	0.268 a^{-}	0.560 $b^{=}$	0.232 b^{+}	0.067 a^{+}	2.522 a^{+}	0.200 a^{-}	0.048 b^{-}	3.383 $b^{=}$	2.653 b^{-}
STS 18 µM	0.216 a^{-}	0.203 c^{-}	0.156 $c^{=}$	0.041 b^{+}	1.206 b^{-}	0.186 a^{-}	0.084 a^{-}	4.051 a^{+}	5.079 $a^{=}$

that H_2O_2 acts downstream from ethylene in *in vitro* senescence and abscission signaling. Therefore, ethylene could induce lipid peroxidation as a result of the increase in H_2O_2 production. Both PODs and PPOs catalysed the oxidation of phenolic substances and are also involved in the phenylpropanoid biosynthesis pathway (Siegel, 1993). A similar result that was observed for PODs was also observed for PPOs (Table 1).

CAT activity was higher than in the control for treatments with $AgNO_3$ and SA (Table 1). The CAT activity observed here supports the hypothesis that $AgNO_3$ and SA are harmful to explant quality, which was reinforced by the highest lipid peroxidation (TBARS) observed when those substances were present (Table 1). On the other hand, reduction in CAT activity was observed when Co was added to the medium (Table 1). In general, under stressed conditions an increase was observed in POD activity, while CAT activity was reduced (Cakmak and Horst, 1991; Siegel, 1993; Agarwal et al., 2005; Molassiotis et al., 2005). This result indicates that H_2O_2 is most highly consumed in oxidative events than removed from the metabolism. We verified an increase in CAT activity (Table 1) especially when more intensive lipid peroxidation was observed (Table 1), reinforcing the hypothesis that $AgNO_3$ and SA increase H_2O_2 production (Mutlu et al., 2009). It is well known that SA may affect the hypersensitivity response as a reaction to the increase of H_2O_2 production (Agarwal et al., 2005), which, in contrast, can be very harmful to *in vitro* culture systems. The observed SOD, POD, PPO and CAT activities (Table 1) suggest that EDTA, STS and mainly Co contribute to the reduction in ethylene production and lipid peroxidation in *in vitro L. filifolia* explants, delaying the onset of senescence in their tissues (Meratan et al., 2009; Vatankhah et al., 2010).

3.4. Pigments contents

The photosynthetic pigment levels in the tissues as well as the relative proportions of them have been used as biomarkers to evaluate different kinds of stresses (Agarwal et al., 2005). The inclusion of Co, EDTA and STS to the culture medium reduced the total content of chlorophyll and carotenoids (Table 1). However, the chlorophyll *a/b* ratio was less affected in response to these substances than to $AgNO_3$ and SA, suggesting an adjustment in stress conditions in the photosynthetic apparatus. Jeon et al. (2006) pointed out that when the chlorophyll *a/b* ratio is kept at levels close to the normal, damages to antenna pigments are generally reduced, suggesting a higher efficiency in the pigments readjustment.

The total anthocyanin content within the tissues varied considerably in response to different treatments (Table 1). The anthocyanin levels remained close to the control in the presence of SA, EDTA and STS, but were reduced when $AgNO_3$ and Co were added to medium. Inhibitory effects of Co on anthocyanin biosynthesis were already reported (Dube et al., 1993). EDTA and etephon, exogenous sources of ethylene, were also associated to flavonoids biosynthesis (Elliot, 1977; Dube et al., 1993). Nagata et al. (2003) suggested that anthocyanin and others flavonoids contribute to the ROS removal. In contrast, Vanderauwera et al. (2005) observed a negative impact of H_2O_2 on DNA clusters transcription related to anthocyanin biosynthesis. Nevertheless, we did not observe any direct relationship here between TBARS and anthocyanin accumulation (Table 1).

Senescence is a natural phenomenon resulting from both the increase of oxidative metabolism and the decrease of antioxidant enzyme activity, and ethylene is particularly associated to these processes (Kumar et al., 1998; Benson, 2000; Ievinsh et al., 2000; Mittler, 2002; Arora et al., 2002; Wang et al., 2002; Meratan et al., 2009). Our study showed that EDTA, STS and mainly Co have protective effects against lipid peroxidation on *in vitro L. filifolia* explants. These protective effects occur, apparently, due to the inhibition effects of those substances on ethylene biosynthesis. The incorporation of EDTA, STS and, mainly Co to the culture medium can effectively contribute to both increasing the efficiency of *L. filifolia* micropropagation and reducing the frequency of subculturing, which have positive effects on *in vitro* germplasm conservation of this species.

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