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BIOLOGY

Effect of salicylic acid and silver nitrate on rutin production by *Hyptis marrubioides* cultured *in vitro*

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ABSTRACT: *Hyptis marrubioides (Lamiaceae) is a medicinal plant that is native from Brazilian Cerrado. In vitro propagation techniques make use of elicitors to alter metabolic pathways, affecting how molecules are produced both qualitatively and quantitatively. This research aimed to evaluate how abiotic elicitors salicylic acid (SA) and silver nitrate (SN) at concentrations of 30μM or 60μM influence Hyptis marrubioides seedling growth by two different in vitro culture methods. The rutin content was quantified by HPLC-DAD. Compared to an untreated culture, the H. marrubioides methanolic extracts cultured in MS medium for 10 days followed by culture in MS medium containing SN (30μM) for 20 days had 1.28 times higher rutin content. In a second experiment, seedlings were cultured in MS medium for 20 days, and then the desired elicitor was added to the culture and allowed to remain in contact with the medium for three and six days. SA (30μM) gave the best results: rutin production was 16.56-foldhigher than the control after six days. SN (30μM) increased the rutin content by 1.17-fold. At the two concentrations evaluated during the elicitation experiments, neither SA nor SN altered the growth parameters shoot length, leaf number, and fresh and dry weight of H. marrubioides seedlings grown in vitro as compared to the control. Based on these results, the abiotic elicitors SA and SN successfully provide Hyptis marrubioides with increased rutin content in vitro.*
Key words: *salicylic acid, HPLC-DAD, tissue culture, silver nitrate, elicitation, plant growth regulators*.

Efeito do ácido salicílico e nitrato de prata na produção de rutina por *Hyptis marrubioides* cultivada *in vitro*

RESUMO: *Hyptis marrubioides (Lamiaceae) é uma planta medicinal nativa do Cerrado brasileiro. Técnicas de propagação in vitro fazem uso de elicitores para alterar as vias metabólicas, afetando a produção de moléculas qualitativa e quantitativamente. Este trabalho teve como objetivo avaliar como os elicitores abióticos ácido salicílico (SA) e nitrato de prata (SN) nas concentrações de 30μM ou 60μM influenciam no crescimento de plântulas de Hyptis marrubioides por dois diferentes métodos de cultivo in vitro. O teor de rutina foi quantificado por CLAE-DAD. Em comparação com uma cultura não tratada, os extratos metanólicos de H. marrubioides cultivados em meio MS por 10 dias, seguidos de cultura em meio MS contendo SN (30*μM) por 20 dias*, apresentaram 1,28 vezes maior teor de rutina. Em um segundo experimento, as plântulas foram cultivadas em meio MS por 20 dias, e então o elicitor desejado foi adicionado à cultura e deixado em contato com o meio por três e seis dias. SA (30*μM) *forneceu os melhores resultados na produção de rutina, sendo 16,56 vezes maior do que o controle após seis dias. SN (30*μM) aumentou o teor de rutina em 1,17 vezes. Nas duas concentrações avaliadas durante os experimentos de elicitação, nem SA nem *SN alteraram os parâmetros de crescimento, como comprimento da parte aérea, número de folhas ou peso fresco e seco das plântulas de H. marrubioides cultivadas in vitro em relação ao controle. Com base nestes resultados, os elicitores abióticos SA e SN forneceram com sucesso Hyptis marrubioides in vitro com maior conteúdo de rutina.*
Palavras-chave: *acido salicílico, CLAE-DAD, cultura de tecidos, elicitação, nitrato de prata, reguladores de crescimento vegetal.*

The genus *Hyptis* belongs to the family Lamiaceae and comprises about 775 species that grow throughout tropical America (MCNEIL et al., 2011). Species of this genus stand out for their economic and ethno-pharmacological importance and have several medicinal applications (BARBOSA and RAMOS 1992).

Hyptis marrubioides, which is native to the Brazilian Cerrado, is known as field-mint and has been used to treat gastrointestinal infections, skin infections, pains, and cramps in folk medicine; its essential oil composition has been extensively studied. (MCNEIL et al., 2011).

Several *in vitro* propagation strategies have been employed to increase the productivity of natural compounds that have great social and economic value (MARASCHIN et al., 2002). One of these strategies has been to use elicitors (chemical, physical, or biological agents) to alter metabolic routes, which affects the resulting molecules qualitatively and

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quantitatively (DJILIANOV et al., 2005; GIRI and ZAHEER2016; RAMIREZ-ESTRADA et al., 2016).

This study has focused on enhancing rutin production in *H. marrubioides* cultured *in vitro*. To this end, two kinds of exogenous elicitors salicylic acid (SA) and silver nitrate (SN) were used at 30µM or 60µM in two different experiments.

H. marrubioides seeds were obtained at the experimental field of the Laboratory of Plant Tissue Culture of Instituto Federal de Educação, Ciência e Tecnologia Goiano, Campus Rio Verde. A voucher specimen (HRV71) was deposited at the Herbarium of the Institute (Herbarium HRV).

The seeds were surface-sterilized with two systemic fungicides—0.2% Bendazol (carbendazim) and 0.2% Alterno (tebuconazole)—for 1 h, subsequently treated with 1% sodium hypochlorite for 30min, and rinsed thrice with sterile distilled water. Next, the seeds were cultured in MS medium (MURASHIGE & SKOOG, 1962) supplemented with 30g L^{-1} sucrose and 3.5g L^{-1} agar; the pH was adjusted to 5.8 before autoclaving. The cultures were maintained in a growth room at an average temperature of 23 ± 1 °C for 30 days, under a 16-hour photoperiod. Two treatments were conducted, and two kinds of exogenous elicitors, salicylic acid (SA) or silver nitrate (SN), at 30µM or 60µM, were employed. For procedure I, the plants were subcultured in glass flasks containing 50mL of MS medium for 10 days and then placed in a new flask containing MS medium and the desired elicitor for 20 days. The control plants were placed in fresh MS medium without elicitor. For procedure II, the plants were subcultured in glass flasks containing 50mL of MS medium. On day 20, the desired elicitor was added to the cultures at the desired concentration and was allowed to remain in contact with the medium for three or six days. Four flasks with five explants were used for each treatment, which amounted to 20 flasks and 100 plants in procedure I including the control group. For procedure II, a total of 40 flasks and 200 plants were used including the control. In both experiments, the plants were maintained in a growth room at 23 ± 1°C, under a 16-hour photoperiod. After treatment with the desired elicitor, the plants were harvested. Next, the shoot length, expanded leaf number, and fresh mass were measured, and the biomass was dried at 35*°C* until constant weight to evaluate the dry mass. The dry *H. marrubioides* plants were extracted with 4mL of HPLC grade methanol by ultrasonic-assisted extraction (30min), filtered through 0.2-µm PTFE filter, and used for HPLC analysis. These procedures were performed in triplicate. The HPLC-DAD analysis for rutin quantitation was carried out on a Shimadzu Prominence LC-20AD binary system equipped with a DGU-20A5 degasser, an SPD-20A series diode array detector, a CBM-20A communication bus module, an SIL-20A HT autosampler, and a CTO-20A column oven (Shimadzu).The stationary phase was a Gemini ODS column (250 × 4.6mm, 5µm; Phenomenex) equipped with a pre-column; the mobile phase was $CH_3OH/H_2O/HOAc$ (5:94.9:0.1, v/v/v) delivered in a linear gradient until 100% CH_3OH was reached within 30 min, followed by 10-min elution with 100% CH_3OH. A total of 20min was allowed for the system to return to the initial conditions. The flow rate was 1.0mL min^{-1};the injection volume was set at 20µL; and UV detection was set at 254nm and 40°C. A standard curve was plotted for different concentrations (0.063mg mL^{-1} to 0.500mg mL^{-1}) of authentic rutin in methanol (HPLC grade, J. T. Baker); each point was measured in triplicate. Rutin was quantified on the basis of the peak area as compared to the rutin calibration curve. The obtained regression equation was $y=4.0 \times 10^7x - 69442$ with a correlation coefficient (R^2) of 0.9998. The external standard rutin was acquired from the standard bank of the Natural Products Group of Universidade de Franca. Statistical analysis was accomplished with the Sisvar 5.3 software (FERREIRA, 2011). The experiment was conducted for each treatment in a completely randomized design with four replications. The averages were compared by the Scott-Knott test at 5% probability.

According to the HPLC-DAD analysis, procedure I, during which the seedlings were cultured in MS medium for 10 days followed by culture in MS medium containing the desired elicitor for 20 days, led to 1.28 times higher rutin content in the *H. marrubioides* methanolic extracts in the presence of SN (30µM) as compared to the untreated culture (Table 1). In contrast, for procedure II, during which the seedlings were cultured in MS for 20 days followed by addition of the desired elicitor for three or six days, SA (30 µM) enhanced rutin production by 16.56-fold as compared to the control, whereas SN (30µM) increased the rutin content by 1.17-fold (Table 1). Our results agreed with the data obtained by PÉREZ et al., (2014) in their study of the effect of chemical elicitors on peppermint (*Mentha piperita*) plants. According to the latter authors, plants treated with SA at 0.5 and 1mM had increased rutin production as compared to non-treated plants (PÉREZ et al., 2014). In the same way, bean sprouts (*Phaseolus vulgaris* L.) treated with SA presented increased rutin content (41-fold) as compared to controls (MENDOZA-SÁNCHEZ et al., 2016). HOU et al. (2015) developed an *in vitro* regeneration system using the buckwheat species *Fagopyrum esculentum* and *Fagopyrum tataricum*. The authors detected the highest rutin content (5.01mg g FW^{-1}) in regenerated plantlets in *F. tataricum* treated

Table 1- Effect of abiotic elicitor type and concentration on rutin production (mg g^{-1} of dry weight) in *Hyptis marrubioides* seedlings.

Treatments	Evaluation time (days)		
	3	6	20
	Rutin teor		
Salicylic acid 30µM	0.3812 ± 0.017[1]A[2]	4.8049 ± 0.321 A	0.1041 ± 0.001 D
Salicylic acid 60µM	0.2392 ± 0.007 E	0.2902 ± 0.009 B	0.1051 ± 0.002 D
Silver nitrate 30µM	0.3483 ± 0.014 B	0.2785 ± 0.045 B	0.2317 ± 0.013 A
Silver nitrate 60µM	0.2742 ± 0.003 D	0.2207 ± 0.009 B	0.1250 ± 0.007 D
Control	0.2958 ± 0.006 C	0.2902 ± 0.0034 B	0.1808 ± 0.003 C
CV(%)	3.66	12.3	4.92

[1]Mean ± standard error.
[2]Means followed by the same uppercase letter in the column do not differ significantly according to the Scott-Knott test at 5% probability.
CV: Coefficient of Variation.

with SA for 24h. In contrast, rutin did not accumulate significantly in *F. esculentum* (HOU et al., 2015).

Elicitation with silver nitrate (SN) also provided a positive response in the case of *Sussurea medusa* suspension cultures with increasing the concentration of the flavones jaceosidin and hispidulin. The maximum jaceosidin and hispidulin yields were 49.90 and 4.95mg L^{-1} after treatment with SN at 0.01mmol L^{-1} on the inoculation day, respectively (ZHAO et al., 2005).

Parameters such as concentration, selectivity, length of exposure to the elicitor, culture age, and nutrient composition can affect the elicitation process (NAMDEO 2007).

Rutin production stimulation might be associated with defense responses promoted by selected elicitors. Indeed, some authors consider that SA is a phytohormone that participates in plant defense reactions, to induce an acquired systemic response, whereas SN can inhibit ethylene biosynthesis. (CURTIS et al., 2004; AL-KHAYRI & AL-BAHRANY, 2001; YELDA et al., 2005).

Compared to the controls, the variables elicitor type, elicitor concentration, and length of exposure to the elicitor did not affect the *H. marrubioides* growth parameters shoot length, leaf number, or average fresh and dry weight during procedures I and II (Table 2 and Table 3).

In contrast, (BRANDÃO, 2014) described decreased growth of *Alternanthere tenella colla* plants treated with AS concentrations of up to

Table 2 - Effect of abiotic elicitor type and concentration on *H. marrubioides* seedling shoot length and leaf number.

Treatments	Evaluation time (days)			Evaluation time (days)		
	3	6	20	3	6	20
	Average shoot length (cm)			Average leaf number		
Salicylic acid 30µM	6.620 ± 0.364[1]Aa[2]	7.500 ± 0.896Aa	4.200 ± 0.287A	14.300 ± 1.526Aa	15.600 ± 1.665 Aa	13.950 ± 0.395A
Salicylic acid 60µM	6.780 ± 0.510Aa	6.475 ± 0.501Aa	4.085 ± 0.389A	14.700 ± 2.068Aa	15.100 ± 1.034Aa	11.771 ± 1.079A
Silver nitrate 30µM	6.845 ± 0.339Aa	7.290 ± 0.547Aa	4.812 ± 0.546A	15.500 ± 1.567Aa	16.800 ± 1.564Aa	11.550 ± 0.715A
Silver nitrate 60µM	5.235 ± 0.304Bb	7.655 ± 0.519Aa	4.800 ± 0.386A	12.350 ± 1.008Aa	16.700 ± 1.382Aa	12.400 ± 0.534A
Control	7.390 ± 0.694Aa	7.405 ± 0.351Aa	5.412 ± 0.481A	14.400 ± 1.071Aa	14.450 ± 0.865Aa	12.775 ± 0.479A
CV(%)	14.54	14.54	25.68	18.62	18.62	14.71

[1] Mean ± standard error.
[2] Means followed by the same uppercase letter in the column do not differ significantly according to the Scott-Knott test at 5% probability.
CV: Coefficient of Variation

Table 3 - Effect of abiotic elicitor type and concentration on *H. marrubioides* seedling fresh and dry weight.

Treatments	----------Evaluation time (days)----------			----------Evaluation time (days)----------		
	3	6	20	3	6	20
	----------Fresh weight (g)----------			----------Dry weight (g) ----------		
Salicylic acid 30 µM	0.289 ± 0.039[1]Aa[2]	0.364 ± 0.071Aa	0.192 ± 0.013A	0.039 ± 0.005Aa	0.042 ± 0.002Aa	0.023± 0.001 A
Salicylic acid 60 µM	0.312 ± 0.025Aa	0.434 ± 0.131Aa	0.263± 0.025 A	0.039 ± 0.004Aa	0.044 ± 0.006Aa	0.026 ± 0.003A
Silver nitrate 30 µM	0.356 ± 0.064Aa	0.336 ± 0.033Aa	0.256 ± 0.039A	0.044 ± 0.005Aa	0.038 ± 0.002Aa	0.027± 0.003 A
Silver nitrate 60 µM	0.284 ± 0.036Aa	0.362 ± 0.051Aa	0.241 ± 0.019A	0.035 ± 0.003Aa	0.042 ± 0.004Aa	0.030 ± 0.002A
Control	0.273± 0.036 Ab	0.504± 0.055 Aa	0.251 ± 0.024A	0.044 ± 0.004Aa	0.051 ± 0.003Aa	0.026 ± 0.002A
CV(%)	34.39	34.39	30.26	19.28	19.28	26.34

[1] Mean ± standard error.
[2] Means followed by the same uppercase letter in the column do not differ significantly according to the Scott-Knott test at 5% probability.
CV: Coefficient of Variation.

400µM. However, explant inoculation followed a different procedure from the protocol used in our study: (BRANDÃO, 2014) inoculated the explant directly in the medium containing the elicitor, whereas we inoculated the explants in fresh medium and only transferred them to the medium containing the elicitor 10 days later. Therefore, in our case, any possible toxic elicitor effect on the plant may have been reduced or avoided because the seedling was already established when it came into contact with SA or SN (BRANDÃO et al., 2014).

These results provide a theoretical basis for the use of the abiotic elicitors SA and SN to favor *H. marrubioides* growth *in vitro* and to obtain increased rutin content in the plant, considering that previous studies on the use of chemical elicitors in *in vitro* cultures of *H. marrubioides* are non-existent.

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DECLARATION OF CONFLICTING INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

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