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ORIGINAL PAPER

Resveratrol biosynthesis on *in vitro* culture conditions in grapevine (*cv.* Feteasca Neagra and Cabernet Sauvignon) under the action of $AlCl_3$ as elicitor agent

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Abstract

Stillbenes are considered the most important phytoalexin group synthesized in the grapevine (Vitis vinifera) and they are known to contribute to the protection against various pathogens. The main typical stillbenes of grapevine, which show antifungal and pharmaceutical characteristics, are resveratrol and his derivatives. Recent studies highlight their benefit for the human health by their antifungal, anticarcinogen, hypolipidemic and antidiabetic properties, besides their already known fungitoxic characteristics. The present study focused upon the induction of resveratrol biosynthesis on in vitro vine culture conditions under the effect of $AlCl_3$ as eliciting agent. The specific medium for vine multiplication (M&S + 1 mg/L BA +0. 5 mg/L IAA) was supplemented with different 1% $AlCl_3$ solution doses (0.01%; 0.03%; 0.05%). The resveratrol dosage was performed through the high performance liquid chromatography (HPLC), using a Merck – Lunchrom pressure liquid chromatograph and a UV detector. A more intensive resveratrol biosynthesis has been observed in the Cabernet Sauvignon variety, where the synthesized resveratrol quantity in the plant was up to 97.94 µg/g d.m. with a 0.05 % $AlCl_3$ concentration in the culture medium.

Keywords: resveratrol, elicitors, (grape-) vine

Introduction

It is known that certain plants can synthesize, in response to stress (UV irradiation or a parasitic infection), natural molecules - generically defined as phytoalexins - enabling them to adapt themselves to this stress [1]. The grapevine produces large quantities of phytoalexins such as polyphenols – the stillbenes being the most important – as a reaction to a physical-chemical stress (UV radiation, ozone) or a biological one (the *Botrytis cinerea*, *Plasmopara viticola*, etc. fungi attack).

The resveratrol, as the main representative of the stillbenes, is synthesized in the leaves or the skin of the grape seeds, becoming a mediator of the plant defense stimulation, thus contributing to the development of a systemic defense of the grapevine [2]. By analogy, the resveratrol has antifungal properties, resisting against the development of microbial infection caused by *Botrytis cinerea*, but also by other pathogens, like *Phomopsis viticola*, *Rhizopus stolonifer* and *Plasmopara viticola*. If all the grapevine varieties of *Vitis vinifera* are capable of producing resveratrol in different quantities, some of them distinguish themselves in its biosynthesis.

Among the chemical agents able to induce the synthesis of resveratrol in the plants that are brought into contact with, the aluminum chloride is the most effective [3, 4]. Our studies meant to determine the active $AlCl_3$doses used for the grapevine multiplication in the *in vitro* conditions.

The objective of this experiment is to identify the varieties designed to provide high quality red wines able to synthesize remarkable amounts of resveratrol, the compound involved in the defense mechanism of the grapevine against the phytopathogenic agents (*Botrytis cinerea, Plasmopara viticola, Phomopsis viticola).*

Materials and Methods

For testing the resveratrol biosynthesis in the *in vitr*o culture, under the action of aluminum chloride, two grapevine clones were selected, designed to provide high quality red wines, namely: Feteasca Neagra 6 St. clone and Cabernet Sauvignon 4 Is. clone. To this end, cultures with additional elicitor agent ($AlCl_3$) in their medium were initiated.

Schematically, the experimental factors are:

A. The $AlCl_3$ doses:

M – Control dose (Romanian **M**artor)

A_1 - 0.01% $AlCl_3$

A_2 - 0.03% $AlCl_3$

A_3 - 0.05% $AlCl_3$

B. The grapevine variety:

B_1 - Feteasca Neagra 6 St. clone

B_2 - Cabernet Sauvignon 4 Is. clone

The biological material (apexes with intense regeneration of 0.3 to 0.5 cm) used for the multiplication through *in vitro* culture, was taken from plants growing in pots under controlled vegetation conditions. The disinfection of the explants was carried out with calcium hypochlorite ($CaCl_2O_2$ 6%), under sterile conditions in laminar flow hood for 5 minutes. The assessment of the regeneration and multiplication processes was carried on the culture medium used for the initiation and multiplication stages and which had in its composition the Murashige -Skoog medium (M & S, 1962), supplemented with 1mg/l N 6-benzilaminopurine (BA) and 0.5 mg/l β-indole acetic acid (IAA). During multiplication, the citokinin /auxin ratio changed to 1:1 (0.5 mg/l BA and 0.5 mg/l IAA), in order to balance the multiplication and elongation processes. Sucrose (20 g/l) was used as a carbon source and agar-agar in the amount of 6.2 g/l, was used for the solidification of the culture media.

According to the general recommendations for the *in vitro* grapevine multiplication, the media pH was adjusted before autoclaving to a value in the range 5.7 to 5.8. The inoculation and transfer operations on fresh media were carried out in sterile areas, in laminar flow hoods.

After stabilizing the cultures, meaning three subcultures (60-65 days), the explants formed adventitious buds which developed into young shoots groups of about 1.5 - 2 cm, under the influence of hormone components in the culture medium. The young shoots selected in these groups were seized in fragments of 0.5-1cm, and were the biological material used for the culture media supplemented with aluminum chloride.

In the experimental variants, the aluminum chloride, in 1% aqueous solution, was added in the culture medium in various doses, the final concentration of the medium in Al^{3+} being of 20 ppm (A_1), 60 ppm (A_2) and 100 ppm (A_3). After neutralization with 10% KOH, the culture medium was sterilized at a temperature of 120^oC in an autoclave for 20 minutes (pressure of 1 at). The regeneration and multiplication processes of the biological material were conducted under controlled conditions, in growth rooms with possibility of adjusting to climate factors (temperature of 25 ± 1^0C; photoperiod and light within 16 hours of light and 3000-3500 lx).

The biosynthesis of the phenolic compounds in the plant material during intermediary metabolism was quantified by determination of the total polyphenols and resveratrol contents

in the experimental variants. The presence of the resveratrol was quantitatively shown by liquid chromatography and UV detection at 306 nm.

Results and Discussions

The observations on the vegetative neo-formations developed from the explants inoculated in the *in vitro* culture, from both grapevine clones, showed that there is uniformity in obtaining the multiplication indices (adventitious buds, number of primary shoots and adventitious shoots) by using the M& S medium as the basic one, improved with 0.5-1mg/l BA and 0.5 mg/l IAA, compared with the medium variants supplemented with aluminum chloride.

The process of the young shoots proliferation was followed periodically. A strong multiplication was remarked in both studied clones (groups of 6-8 primary shoots/young shoots fragment) and a clear elongation in the A_1 variant, with the lowest aluminum salt content (0.01%). The multiplication was lower for the other two experimental variants, but with increased ascension of the young shoots.

After 20 days of tissue culture, Feteasca Neagra 6 St. clone expressed a better multiplication rate in variant A_1 (0.01% $AlCl_3$) compared with the same variant, but with Cabernet Sauvignon 4 Is. clone.

At higher concentrations of aluminium (60 ppm and 100 ppm Al^{3+}) in the culture medium the rate of multiplication decreased, the elongation being favored for the young shoots. For the A_2 but especially A_3 variants, a slight reddening of the leaves was also observed, due to the anthocyanin biosynthesis.

The chemical analyses conducted on the collected material from the experimental variants showed an increased content of total polyphenols and of resveratrol in the plants grown on the medium containing the elicitor agent.

For the Feteasca Neagra variety, the highest $AlCl_3$ concentration in the culture medium led to a significant increase in total polyphenols content in the grapevine plants (Figure 1 A, B).

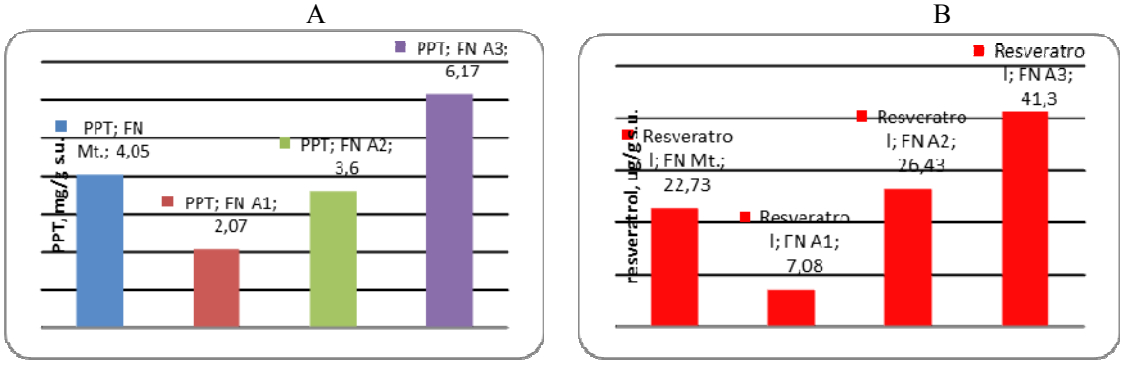
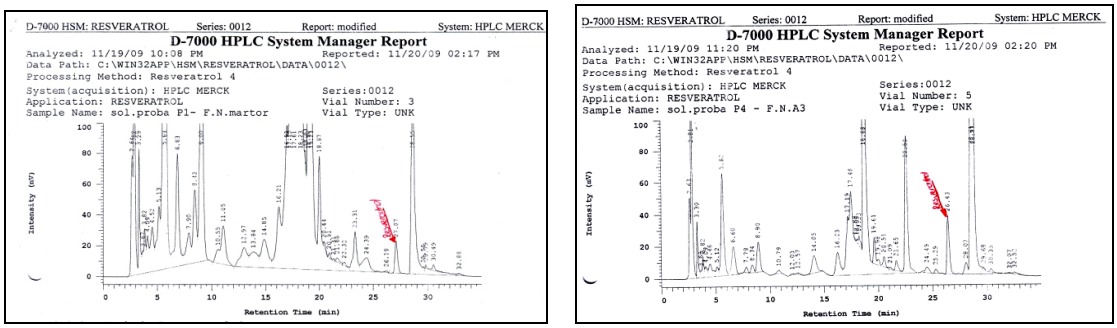


Figure 1. Total polyphenols (A) and resveratrol (B) content in *in vitro* grapevine multiplied plants (B1 - Feteasca Neagra 6 St. clone) on different media (A_1, A_2, A_3).

Similarly, the same content of 0.05% elicitor agent in the culture medium almost doubled the content of resveratrol in A_3 variant, compared with the control variant (Figure 2).



(a) (b)

Figure 2. Chromatograms with trans-resveratrol content in control plants (a) and plants growing on A_3 media variant (b) – Feteasca Neagra 6 St. Clone

The variety of Cabernet Sauvignon 4 Iş clone had a better response to the treatment with $AlCl_3$ in comparison with Feteasca Neagra. So, both total polyphenols and resveratrol content values were directly correlated with increased doses of elicitor added in the culture medium (Figure 3). Furthermore, the resveratrol biosynthesis was increased in this variety, compared with the variety of Feteasca Neagra 6 St. clone. Thus, the resveratrol content reached 97.94 µg/g d.m. in the A_3 variant (Figure 4), compared to 41.3 µg/g d.m., recorded in the similar variant of the variety Feteasca Neagra.

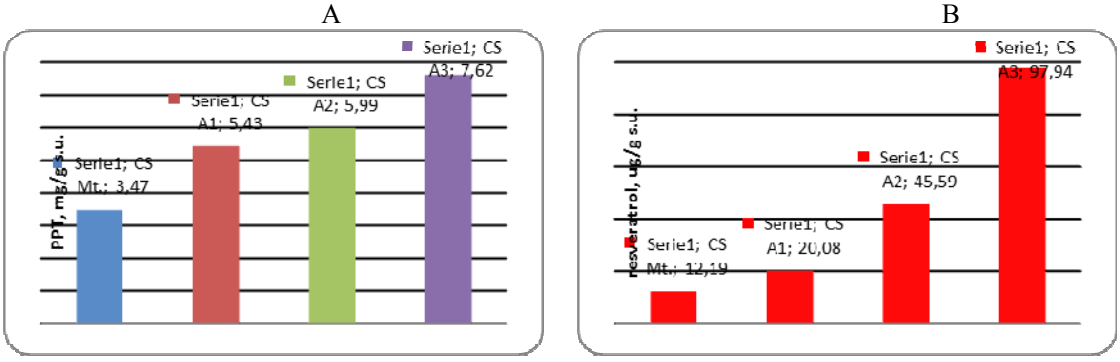
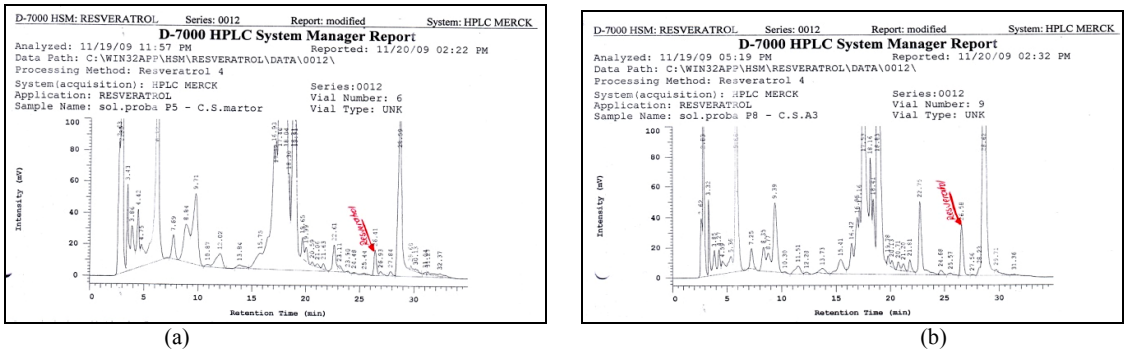


Figure 3. Total polyphenols (A) and resveratrol (B) content in *in vitro* multiplied plants (B2 - Cabernet Sauvignon 4 Is. clone) on media with different doses of $AlCl_3$ (A_1, A_2, A_3)



(a) (b)

Figure 4. Chromatograms with trans-resveratrol content in control plants (a) and plants growing on A_3 media variant (b) – Cabernet Sauvignon 4 Is. clone

It is remarkable that in the plants belonging to the control variant, the resveratrol content was significantly higher in the Feteasca Neagra variety compared to the Cabernet Sauvignon one.

Conclusions

To highlight the influence of aluminum chloride in the biosynthesis of resveratrol on *in vitro* culture conditions of the grapevine, a laboratory procedure was initiated and the $AlCl_3$ 1% solution was added in the culture medium in various doses;

The tested varieties responded to the addition of the elicitor agent in the culture medium by showing an increase of the total polyphenols content, but especially of the resveratrol one, according to the used dose;

The resveratrol biosynthesis occurred with greater intensity in Cabernet Sauvignon 4 Is. clone compared with Feteasca Neagra 6 St. clone, the amount of resveratrol synthesized in the plants reaching the value of 97.94 μg/g d.m., at a concentration of 0.05% $AlCl_3$ (100 ppm Al^{3+}).

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