The impact of Ceftriaxone on Relative Copy Numbers of Mitochondrial and Chloroplast DNA in Grapevine Leaves

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Abstract. The cultivation of micropropagated grapevine plants *in vitro* is currently undergoing improvements in existing methods and the development of new cultivation techniques due to the problem of bacterial contamination caused by multidrug-resistant microorganisms. Explants (*Vitis vinifera* L., 'Chardonnay' variety) were cultured on Murashige-Skoog basal medium supplemented with different concentrations of ceftriaxone: 0 mg/L, 250 mg/L, and 1000 mg/L. After 30 days, morphometric characteristics of the micropropagated plants and relative copy numbers of mitochondrial and chloroplast DNA were evaluated. Leaf samples (5-10 mg) were randomly selected from each plant group for subsequent total DNA extraction. Quantitative RT-PCR was performed using LightCycler 480 SYBR Green I Master Mix (LifeScience, Roche) and analyzed with a LightCycler 96 automated analyzer (Roche Life Science). The relative copy numbers of NAD1 (mitochondrial DNA) and rps16 (chloroplast DNA) genes were determined using the GAPDH gene (chromosomal DNA) as the reference. The 2^{-DCt} and 2^{-DDCt} algorithms were used for quantitative assessment. Ceftriaxone at concentrations of 250 and 1000 mg/l reduces the relative number of copies of chloroplast and mitochondrial DNA, which indicates the suppression of photosynthesis and oxidative phosphorylation in grape microplants. The experimental scheme developed by us can be successfully used as a test system for assessing the degree of influence of various biogenic and abiogenic factors on plant objects in order to optimize their cultivation.

1 Introduction

Grapevine *in vitro* propagation is currently in the stage of improving existing and developing new cultivation methods [1]. Bacterial contamination of eukaryotic cell cultures (BCE) caused by multidrug-resistant microorganisms is reported worldwide. Since BCE is associated with reduced viability and productivity of the obtained bioproducts, it is essential to control and prevent bacterial infections at an early stage using appropriate antibiotics. Ceftriaxone remains the drug of choice for preventing BCE. High prevalence of resistance to commonly used antibiotics, such as ampicillin (94.9–90.7%), cefotaxim (92.4–71.4%),

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piperacillin-tazobactam (31.2–27.5%), and levofloxacin (42.4–39.8%), has been observed [2]. Carbapenem resistance is also observed worldwide, ranging from 1% to 79%, with *K. pneumoniae* and *Acinetobacter baumannii* Bouvet & Grimont showing the highest antibiotic resistance [2].

Since the establishment of cell, tissue, and organ cultures, antibiotics are widely used either as selective markers for genetic transformation (kanamycin, paromomycin, hygromycin, etc.) or for eliminating any bacterial contamination. Recently, antibiotics from the group of β-lactams, such as timentin and ceftriaxone, have been used more frequently [3]. Researchers using antibiotics with plant cultures report their influence on plant morphometric parameters. In some cases, ceftriaxone and other antibiotics inhibit plant growth [4], while in others, they enhance it [5]. Although β-lactam antibiotics are considered non-toxic to plants, many researchers note that their influence on growth and morphogenesis can significantly vary depending on the plant species and antibiotic concentration [6].

Beta-lactamases (AmpC) mediate resistance to cephalothin, cefazolin, cefoxitin, most penicillins, and beta-lactam/inhibitor combinations. Overexpression of AmpC confers resistance to broad-spectrum cephalosporins, including cefotaxim, ceftazidime, and ceftriaxone, and presents a problem, especially in infections caused by *Enterobacter aerogenes* Hormaeche and Edwards and *E. cloacae* Hormaeche and Edwards, where initially susceptible isolates may become resistant to these agents. Transmissible plasmids have acquired AmpC enzyme genes, and therefore, they can appear in bacteria lacking or poorly expressing the chromosomal gene bla (AmpC), such as *Escherichia coli* Castellani and Chalmers, *K. pneumoniae*, and *Proteus mirabilis* Hauser. AmpC enzymes, encoded by both chromosomal and plasmid genes, evolve to more efficiently hydrolyze broad-spectrum cephalosporins. Relative copy number parameters of mitochondrial and chloroplast DNA closely correlate with gene expression characteristics of these organelles and can, therefore, be used as indicators of their physiological state [7].

With this in mind, the aim of our study was to investigate the potential effects of ceftriaxone at various concentrations on the parameters of relative DNA copy numbers of mitochondrial and chloroplast DNA in grapevine leaves *in vitro*.

2 Materials and Methods

At the initial stage of the model experiment, all explants (*Vitis vinifera*, 'Chardonnay' variety) were cultivated on a Murashige and Skoog (MS) basal medium supplemented with 30 g/l sucrose, 0.2 mg/l indole-3-acetic acid (IAA), and 2 mg/l 6-benzylaminopurine (6-BAP) [8]. In each of the experimental vessels with the nutrient medium, 10 grape explants were introduced. Subsequently, the experimental vessels with the nutrient medium and explants were divided into three groups:

1. Control group of plants, grown on MS nutrient medium without the addition of ceftriaxone.
2. Experimental group of plants, grown on MS nutrient medium with a ceftriaxone concentration of 250 mg/l.
3. Experimental group of plants, grown on MS nutrient medium with a ceftriaxone concentration of 1000 mg/l.

Plants from all experimental groups were combined and cultivated at a temperature of 23 ± 10°C with a 16-hour photoperiod and illumination provided by 40-watt cold white fluorescent lamps with an intensity of 105–115 μmol PPFD/m²/s (PPFD = photosynthetic photon flux density) for 30 days. Leaf samples (5–10 mg) were randomly selected from each group of plants (control and with ceftriaxone concentrations of 250 and 1000 mg/l) for subsequent total DNA extraction [8].

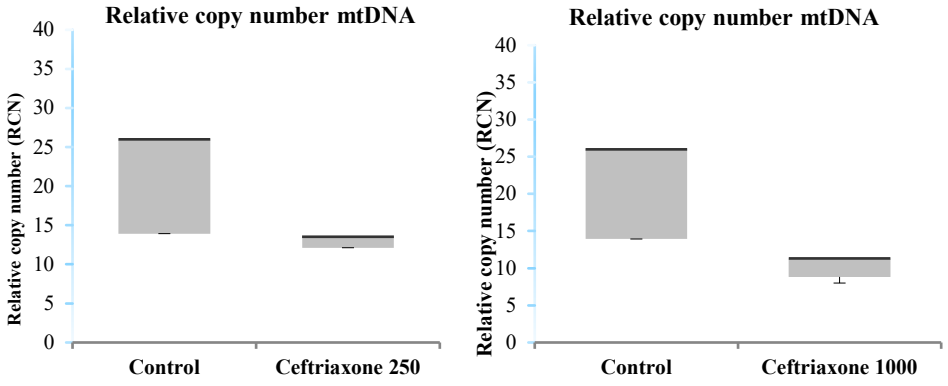
Quantitative RT-PCR was conducted in a 20 µl reaction mixture, consisting of 10 µl of LightCycler 480 SYBR Green I Master Mix (LifeScience, Roche, Penzberg, Germany), 5 ng of DNA (5 µl), 3 µl of water, and 1 µl of corresponding primers (forward and reverse, 0.33 µM). RT-PCR was performed using an automatic analyzer, LightCycler 96 (Roche Life Science), with the following program: initial denaturation at 95°C for 5 minutes (1 cycle), followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 25 seconds, and extension at 72°C for 25 seconds. The relative copy numbers of the NAD1 (mitochondrial DNA) and rps16 (chloroplast DNA) genes were determined using the GAPDH gene (chromosomal DNA) as a reference. Quantitative assessment was carried out using the 2-DCt and 2-DDCt algorithms [9].

Table 1. Primers Used in the Study

Primer name	**Nucleotide sequence 5′→3′**	**Amplicon length**	**Tm (^{0}C)**	**Cellular compartment**
***GAPDH*_F**	CGA CAG TGT TCA CGG TCA GT	**85**	**60**	Nuclear DNA
***GAPDH*_R**	GGT GAC TGG CTT CTC ACC AA			
***RPS16*_F**	CGG ATC ATA AAA ACC CAC TTT CCG	81	60	Chloroplast DNA
***RPS16*_R**	GCC GTC TAT CGA ATC GTT GC			
***NAD1*_F**	GGC TCA TTC TCC AAA CGG GA	73	60	Mitochondrial DNA
***NAD1*_R**	CCT ATG GCC GAT CTG TCA CC			

Statistical analysis was performed using Statistica 13.3.0 software (TIBCO Statistica, 2017) with default parameters. The statistical significance of differences between groups was assessed using the non-parametric Mann-Whitney test and Fisher's F-test.

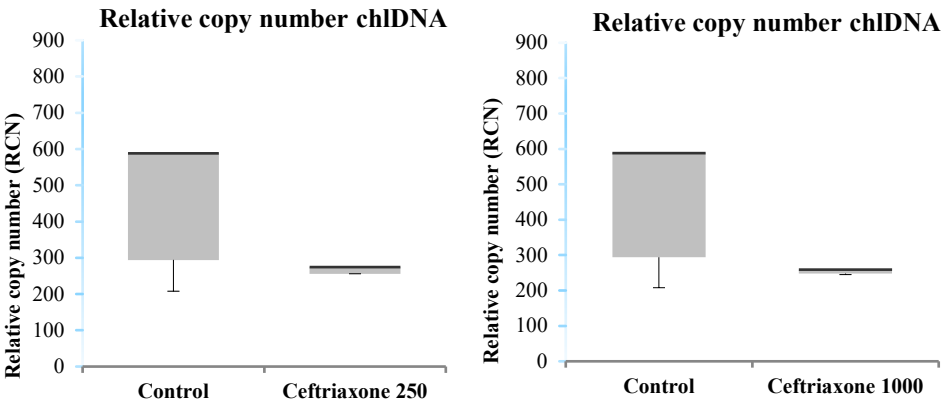
3 Results and discussion



a) b)

Fig. 1. Changes in the relative number of copies of mitochondrial DNA (mtDNA) in grape leaves after adding ceftriaxone to the MS nutrient medium at concentrations of 250 mg per 1 L of MS nutrient medium (a) and 1000 mg per 1 L of MS nutrient medium (b). Median values. The reference gene for comparison is *GAPDH* (nuclear DNA).

As follows from our experimental data, the median values of the relative number of copies of mitochondrial DNA in grape leaves in the group of control plants were 26 copies, and in the leaf tissues of plants grown on MS medium with a ceftriaxone concentration of 250 mg/l - 13.5 copies (Figure 1a). Our data clearly demonstrated a decrease in the relative copy number of mitochondrial DNA in grape leaves when grown on MS medium with a ceftriaxone concentration of 250 mg/l by 48.1% compared with similar indicators in the group of control plants. This indicates that ceftriaxone at a concentration of 250 mg/l causes a statistically significant (p=1.2%, Mann-Whitney test) decrease in the copy number of mitochondrial DNA in grape leaves and, therefore, the expected inhibition of oxidative phosphorylation processes in grape leaves. When the concentration of ceftriaxone in the MS nutrient medium increases to 1000 mg/l (Figure 1b), the processes of decreasing the relative copy number of mtDNA in grape leaves intensify: the relative mtDNA copy number indicators at a ceftriaxone concentration in the MS nutrient medium of 1000 mg/l are 54% less than the same control indicators (Figure 1b). These differences are statistically significant at a significance level of p=0.7% (Mann-Whitney test).



a) b)

Fig. 2. Changes in the relative copy numbers of chloroplast DNA (chlDNA) in grape leaves after adding ceftriaxone to the MS nutrient medium at concentrations of 250 mg per 1 L of MS nutrient medium (a) and 1000 mg per 1 L of MS nutrient medium (b). Median values. The reference gene for comparison is *GAPDH* (nuclear DNA).

Solar energy is efficiently utilized by plants for energy accumulation and the synthesis of organic compounds. This process, called photosynthesis, is localized in organelles known as chloroplasts. The quantity of chloroplast DNA copies reflects the intensity of these organelles' work [10-11]. Therefore, in this section of our study, we investigated the potential impact of different concentrations of ceftriaxone (250 and 1000 mg/l) in the MS medium on the efficiency of chloroplasts' function, as assessed by the relative copy numbers in the leaves of *in vitro* cultured microplants of 'Chardonnay' grapevine. As evident from our experimental data presented in Figure No. 2, ceftriaxone at a concentration of 250 mg/l in the MS medium inhibits the relative copy numbers of chloroplast DNA by 53.3% compared to control values: if the control values of relative chloroplast DNA copy numbers are 588.1 copies, then after growing microplants on the medium with a ceftriaxone concentration of 250 mg/l, this value is 274.4. The differences between these values are statistically significant at a significance level of p=0.1% (Mann-Whitney test). This allows us to assert with a high degree of certainty that ceftriaxone at a concentration of 250 mg/l in the medium exerts an inhibitory effect on the function of chloroplasts in microplants of 'Chardonnay' grapevine in conditions *in vitro*. Similarly, based on our data presented in Figure No. 2b, ceftriaxone at a concentration of 1000 mg/l also has a pronounced inhibitory effect on the relative copy numbers of chloroplast DNA by 56%: if the control values of relative chloroplast DNA copy numbers are 588.1 copies, then after growing microplants on the medium with a ceftriaxone concentration of 1000 mg/l, this value is 259 copies. The differences between these values are statistically significant at a significance level of p=0.7% (Mann-Whitney test).

Thus, it can be concluded that ceftriaxone at concentrations in the MS medium of 250 mg/l and higher exerts an inhibitory effect on the function of chloroplasts and mitochondria in microplants of 'Chardonnay' grapevine, as assessed by the relative copy numbers of their DNA. Statistically significant changes in the ratios of relative copy numbers of chloroplast DNA to similar mitochondrial DNA values were not observed at different ceftriaxone concentrations.

4 Conclusions

1. The introduction of ceftriaxone into the MS medium for growing grapevine microplants *in vitro* conditions leads to a reduction in the relative copy numbers of mitochondrial DNA by approximately 50%. This indicates the possible suppression of oxidative phosphorylation processes in grapevine tissues by ceftriaxone at the examined concentrations (250 and 1000 mg/l).

2. Cultivating 'Chardonnay' grapevine microplants *in vitro* on MS medium with the addition of ceftriaxone at concentrations of 250 and 1000 mg/l results in a statistically significant reduction in the relative copy numbers of chloroplast DNA by approximately 50%. This suggests the inhibition of photosynthesis by ceftriaxone at the examined concentrations (250 and 1000 mg/l).

3. The significant suppression of relative copy numbers of mitochondrial and chloroplast DNA by ceftriaxone at the examined concentrations (250 and 1000 mg/l) indicates a general inhibition of metabolic processes in 'Chardonnay' grapevine microplants.

4. Ceftriaxone in the MS medium at a concentration of 250 mg/l increases the number of leaves on grapevine microplants by approximately 50%, while at a concentration of 1000 mg/l, it reduces their number by approximately 30% compared to control values.

One possible explanation for the observed inhibitory effects of ceftriaxone on intracellular organelles in grapevines (mitochondria and chloroplasts) is their endosymbiotic origin from bacterial ancestors, namely α-proteobacteria and cyanobacteria [10].

The experimental scheme developed by us can be successfully applied as a test system for assessing the impact of various biogenic and abiotic factors on plant organisms.

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