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TRABALHO DE CONCLUSÃO DE CURSO

Acúmulo de saponinas bioativas em *Quillaja brasiliensis* induzido por irradiação Ultravioleta-C e luz vermelha no período pós-colheita

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Accumulation of bioactive saponins in detached leaves of *Quillaja brasiliensis* induced by Ultraviolet-C radiation and red light irradiance: a comparative analysis

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Abstract

Saponins are terpene metabolites found in several plant species. A fraction of triterpene saponins from leaves of *Quillaja brasiliensis,* named QB-90, has shown great potential as immunoadjuvant in vaccines, constituting a sustainable source of metabolites with this useful bioactivity. It has been shown that higher concentrations of these saponins can accumulate in detached leaves of young plants by exposure to red light or UV-C irradiation. These studies have used frames with cellophane filters to filter the fluorescent light output and enrich for red light irradiance reaching leaves. The present study aimed at evaluating if LED lamps can be viable alternatives to cellophane filters as sources of red irradiance for treating detached leaves to increase QB-90 concentration, as well as to examine the effect of combined exposure of UV-C and red light on bioactive saponin yield. The source of leaves for the present experiments was 11 month-old seedlings of *Q. brasiliensis*, cultivated under controlled conditions. Both red light enrichment methods, LED lamps or cellophane filter, had the same stimulatory effect on QB-90 accumulation. The combined treatment of 15 minutes of UV-C followed by 11 h and 45 min of red light irradiance promoted QB-90 accumulation, but did not show significant differences when compared to 12 hours of red light treatment or 15 minutes of UV-C followed by 11 hours and 45 min of white light irradiation treatment. The treatment combining red light with 3 UV-C pulses yielded no increase of the QB-90 concentration in comparison to the control. Therefore, red light, provided by LEDs, or UV-C exposure for 15 min are the best choices of post-harvest treatment to increase QB-90 concentration in leaves.

Keywords

Saponin, *Quillaja brasiliensis*, LED, red light, UV-C, post-harvest, QB-90

1. Introduction

Saponins are a class of terpenoids whose main structure (which can be triterpenic or steroidal) is conjugated with one or more sugar residues. They can be found in many species of plants. However, as secondary metabolites, which are not essential for the plant survival in optimal conditions but are of great adaptive value under natural conditions, saponins are often found in low concentrations. Secondary metabolites have several different functions in plants, such as defence against herbivores and pathogens, protection against ultraviolet (UV) radiation and low temperatures, attraction of pollinators, among other functions (Sandes & Blasi, 2000). Saponins can present different properties, including hemolytic and ichthyotoxic action, and display an amphiphilic character, forming foam in water (Vincken et al., 2007). They are widely used in food, pharmaceutical, cosmetic and textile industries due of their properties. Regarding pharmaceutical use, the adjuvant activity of saponins in vaccines is very pronounced, being obtained mainly from roots of *Panax ginseng*, *P. notoginseng*, *Platycodon grandiflorum*, *Polygala senega* and *Quillaja saponaria* (De Costa et al., 2011). In case of *Q. saponaria*, saponins are obtained from the barks of trees between 30 to 50 years old. This method of obtaining the metabolites is destructive and can lead to forest depletion, demanding around 28.000 adult plants per year to supply the current demand.

The Chilean exports of “extract of quillay” from *Q. saponaria* moved more than 9 billion dollars in 2014, the main importing countries being Brazil and the United States of America (ProChile, 2015). In the European Union, “Quillaia extract” is already regulated as a food additive under the code “E999”, and is commonly used as a foaming agent in soft drinks (Food Standards Agency, 2014).

As an alternative to *Q. saponaria* exploitation to obtain saponins, there is a related species called *Quillaja brasiliensis*, native from Brazil, Uruguay and Argentina. Previous studies showed that the saponin enriched fraction from *Q. brasiliensis*, known as QB-90, has the same immunoadjuvant efficiency in vaccines against *poliovirus* and bovine *herpesvirus* types 1 and 5 as the commercial fraction of *Q. saponaria* known as Quil-A® (De Costa et al., 2014; Fleck et al., 2006; Silveira et al., 2011). Additionally, QB-90 is obtained in larger concentrations from the leaves of young plants, being a more sustainable form of exploitation than removing barks of slow growing trees. It is already known that the production of saponins in leaves of *Q. brasiliensis* is modulated by abiotic external conditions such as water potencial and irradiance, and by biotic stress factors or simulations of these, such as herbivory, pathogen attack, mechanical wounding and ultrasound application (De Costa et al., 2013). More specifically, it was found that QB-90 can be induced by UV-C or red light irradiance in direct exposure to the plant or in detached leaves (Yendo et al., 2015).

Irradiance can be correlated to the stimulation of various secondary metabolites, such as gingerol and zingiberene in *Zingiber officinale* when compared to continuous dark cycle (Anasori and Asghari, 2008). Different qualities of irradiation or light intensity have been tested to analyze their effects in secondary metabolites regulation and production (Ramakrishna and Ravishankar, 2011). Arakawa (1985) reported a synergistic stimulation in anthocyanin production using simultaneous irradiation of red light and UV-B on apple fruits skin disks.

A recent study with *Q. brasiliensis* has shown a pronounced accumulation of QB-90 in detached leaves of young and adult plants using UV-C or red light irradiation (Yendo et al., 2015). This study used cellophane frames to obtain enrichment of red irradiance on detached leaves by filtering the output of white fluorescent tubes. However, in an industrial scale this method may not be optimal due its maintenance demands, which include the regular replacement of the filters to ensure irradiance quality. Light-emitting diodes (LEDs) have been used as substitutes for fluorescent and sodium-vapor lamps due of their higher spectral composition control and high light output with low radiant heat (Morrow, 2008). In spite of the relatively high cost of LED lamps, they have longer lives and use less energy to produce the same irradiance as conventional lamps, being regarded as a promising technology for the greenhouse industry (Olle, 2013).

Based on the potential of sustainable exploration of *Q. brasiliensis* and on the good market value and increasing demand of saponins, the establishment of optimal conditions for bioactive saponin production is a relevant goal to pursue. Thus, this study was conducted to investigate the effectiveness of red light LED lamps compared to red cellophane frame filters to induce QB-90 accumulation in detached leaves of *Q. brasiliensis* young plants, as well as to evaluate the response of saponin concentration in detached leaves to combinations of UV-C and red light irradiation exposure.

2. Materials and methods

2.1. Plant material

Seeds from *Q. brasiliensis* were collected from adult tree plants in Canguçu, RS, Brazil (31°23'42''S , 52°40'32''W). A voucher specimen is deposited at the UFRGS University Herbarium (ICN 142953). Seeds were stored at 10 ± 2 °C for 24 hours before germination. Then, the seeds were submitted to surface sterilization with Dithane® (antifungal agent) 5 g L^{-1} for 20 min, followed by one min of immersion in 70% ethanol and 15 min in sodium hypochlorite solution (2.5%) with a few drops of neutral detergent. Then, seeds were washed three times in sterile distilled water. After surface disinfestation, the seeds were germinated, as previously described (Fleck et al., 2009). After two months, germinated plants were transferred to *ex vitro* conditions, in plastic pots containing previously autoclaved vermiculite, and receiving water and nutrient solution twice a week. Pots were maintained in a growth room under white

fluorescent tubes at 25 ± 2 °C in a 16/8 h day/night cycle, with photosynthetically active radiation (PAR) of approximately 45 µmol m^{-2} s^{-1}, until the experimental period. Fully expanded leaves were collected from 47 shoots of juvenile individuals with approximately eleven months, superficially cleaned, and submitted to the treatments as described below (2.2 and 2.3). For the determination of the basal concentration of QB-90 in leaves, prior to the experiments, 6 samples with biological triplicates (three leaves per sample) were collected and frozen in liquid nitrogen and stored at -80 °C until extraction.

2.2. Comparison between red LED lamps and red cellophane filters on QB-90 accumulation in leaves of young plants

2.2.1. Cellophane filter treatment

Detached leaves were kept in Petry plates, in a growth chamber at 25 ± 2 °C in a 16/8 hours day/night cycle, irradiated with white fluorescent tubes (PAR 45 µmol m^{-2} s^{-1}), and covered with a red cellophane filter frame for plants (allowing plenty of gas exchange), resulting in an enrichment of the transmitted light in the red range (total PAR normalized to 35 µmol m^{-2} s^{-1}). A transparent cellophane filter was used as control. Spectral characteristics of filter output have been previously described (Ruedell et al., 2013). Leaves were sampled 12 h after the beginning of the experiment. After harvest, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction.

2.2.2. Red LED treatment

Detached leaves kept in a growth chamber at 25 ± 2 °C in a 16/8 hours day/night cycle, were placed on Petry plates and submitted to red light irradiance of LED lamps (Sincrona® red LED PAR30 3,1W; PAR 45 µmol m^{-2} s^{-1}). Leaves were collected 12 h after the beginning of the experiment. After harvest, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction.

2.3. Effect of red irradiance using LEDs enrichment combined with UV-C exposure on detached leaves of young plants

2.3.1. UV-C radiation followed by red light irradiation treatment

Detached leaves kept in a growth chamber at 25 ± 2 °C in a 16/8 hours day/night cycle were placed on Petry plates and submitted to UV-C light (germicide lamp, λ max 254 nm, 10.5 kJ cm^{-2}) in a closed chamber for 15 minutes and then placed under red light (PAR 45 µmol m^{-2} s^{-1}) for additional 11 h and 45 min. Leaves were collected 12 h after the beginning of the experiment. After harvest, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction.

2.3.2. Red light irradiation with UV-C light pulses

Detached leaves kept in a growth chamber at 25 ± 2 °C in a 16/8 hours day/night cycle were placed on Petry plates and submitted to red light (PAR 45 µmol m^{-2} s^{-1}) and after approximately 4 hours placed under UV-C light for 15

minutes. This was repeated three times, so that the leaves received three pulses of UV-C interlaced with red light irradiance. Leaves were collected 12 h after the beginning of the experiment. After harvest, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction.

2.3.3. UV-C treatment

Leaves were placed in Petry plates, in a closed chamber and exposed to UV-C light for 15 minutes. After that, they were placed under white light from fluorescent tubes (PAR 45 µmol m^{-2} s^{-1}) for additional 11 hours and 45 minutes. Leaves were collected 12 h after the beginning of the experiment. After harvest, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction.

2.3.4 Red light treatment

Detached leaves kept in a growth chamber at 25 ± 2 °C in a 16/8 hours day/night cycle, were placed on Petry plates and submitted to red light (PAR 45 µmol m^{-2} s^{-1}). Leaves were collected 12 h after the beginning of the experiment. After harvest, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction.

2.3.5 Control

Detached leaves kept in a growth chamber at 25 ± 2 °C in a 16/8 hours day/night cycle, were placed on Petry plates and submitted to white light of fluorescent tubes (PAR 45 µmol m^{-2} s^{-1}). Leaves were collected 12 h after the beginning of the experiment. After harvest, leaves were frozen in liquid nitrogen and stored at -80 °C until extraction.

2.4. Determination of QB-90 content

Extraction of samples was performed by grinding leaves in mortar and pestle with liquid nitrogen. Then, samples were macerated with distilled water (proportion 1:40, w/v) for three minutes, followed by ultrasonication for 40 minutes. Extracts were filtered, lyophilized and analyzed in a Thermo Scientific® HPLC, following a previously described method (Fleck et al., 2012). Chromatography was performed on a Waters Spherisorb® C8 reverse phase column 150 x 4.6 mm, with corresponding guard column, using an isocratic system with acetonitrile and water (65:35). Mobile phase was previously filtered through Millipore® membranes (0.22 µm) and degassed. Flow rate was 0.8 mL min^{-1} and detection was done at 214 nm. To quantify QB-90, 25 µL of samples were injected and an external standard curve was generated using authentic QB-90 purified and isolated from leaves, according to De Costa et al. (2011). The identification of fraction QB-90 was done by analyzing the retention time and co-chromatography with authentic QB-90 standard, as described by Fleck et al. (2012). The contents of QB-90 in samples were expressed as percentage of extracted dry weight.

2.5. Statistical analyses

All assays herein described were performed in totally randomized layout, with biological triplicates. In each assay, all treatments were carried out in

sextuplicate. All data on QB-90 irradiance-based induction experiments were analyzed by ANOVA followed by Duncan test. For all tests, we used P-value ≤ 0.05 and the statistic package SPSS 17.0 for Windows. Results were expressed as the mean, plus or minus standard errors. A assay repetition is still needed to be done to reinforced the results.

3. Results

3.1. Comparison between red LED lamps and red cellophane frame filter on QB-90 accumulation in leaves of young plants

The detached leaves derived from young plants exposed to red light provided by LED lamps or cellophane filter induced the production of QB-90 when compare to the control (Figure 1), without showing significant differences between the red light sources (average of QB-90 and standard deviation: control = 0.427 ± 0.018 % DW; LED = 0.576 ± 0.018 % DW; cellophane = 0.537 ± 0.024 DW).

3.2 Effect of red irradiance enrichment combined with UV-C exposure on detached leaves of young plants

Detached leaves exposed to enriched red irradiance, UV-C treatment and UV-C followed by red light treatment had significantly higher content of QB-90 (control: 0.454 ± 0.024 % DW; red: 0.642 ± 0.047 DW; UV-C: 0.712 ± 0.059 % DW; UV-C plus red: 0.776 ± 0.092 DW). Enrichment with red light with UV-C

pulses did not cause significant change in the yield of QB-90 (red plus UV-C pulses: 0.457 ± 0.028 % DW) (Figure 2).

4. Discussion

Since the invention of LEDs, many studies have been conducted in order to evaluate its performance and effect on plant growth. LEDs have been shown to be an important tool in research for its spectral control quality, low profile and low radiant heat output (Morrow, 2008). Although costs are still relatively high, the useful life of LEDs is relatively long and there are predictions on price reduction and further increase in LEDs' efficiency over the coming decade (Olle, 2013). Under these circumstances, it seems very suitable to determine if LED is efficient for the induction of saponins in *Q. brasiliensis* for future commercial applications. The results show that LED is as efficient as the cellophane filter used in previous studies. The results with LED showed an even more homogeneous induction with a lower standard deviation than cellophane and its mean values, although not statistically different, are slightly higher in absolute terms.

For many years studies that associate red light irradiation with induction of different kinds of secondary metabolites have been reported, including reports with flavonoids, anthocyanins, carotenoids, and monoterpenes (Liu et al., 2009; Yamaura et al., 1991). Also recent studies with UV-C light on post-harvest fruits, such as blueberries, apples and grapes, have shown an increase of the

antioxidant activity and flavonoid content (Zoratti et al., 2014). Our results ascertain these previously studies and show an increase on the QB-90 content when detached leaves are exposed to 12 hours of red light and when these are exposed to 15 minutes of UV-C followed by white light. These results also corroborate the findings of Yendo et al. (2015) that showed the same kind of content increase in leaves of young plants. Yendo et al. found that UV-C irradiation induces significantly more QB-90 than red light irradiation, whereas our results showed similar concentrations for both treatments. However, Yendo et al. (2015) harvested leaves right after 15 min of UV-C exposure, whereas, in the present experiments, leaves were kept under white light until the completion of a total period of 12h after the 15 min UV-C treatment. Hence, it appears that UV-C can cause an acute induction of QB-90, which partially fades over time, perhaps acting as a quencher for reactive oxygen species generated upon UV-C treatment (Yendo et al., 2015).

Regarding the result of the UV-C followed by red light treatment, there was no synergistic effect observed; instead, a statistically equivalent induction of QB-90 was observed in the combination of UV-C followed by red light, in the red light only and UV-C only treatments. Nevertheless, by analysing the raw data it is possible to observed a slight increase in QB-90 content in the UV-C plus red light treatment. Red light treatment had a mean increase of 188 mg / 100 g DW in relation to the control, UV-C increased 257 mg / 100 g DW and UV-C plus red light increased 322 mg / 100 g DW. That could be a indication that red light and UV-C light could present an additive effect on the QB-90 content induction in other schemes of combined exposure.

The result of QB-90 accumulated in the red light with UV-C pulses treatment was as low as that displayed by the control. This result suggests that the amount of UV-C irradiation that the leaves were exposed to was excessive, leading to cell damage, possible due to impairment or de-regulation of saponin metabolism, increasing catabolism as proposed by De Costa et al (2013) in a previous study with UV-C irradiation on *Q. brasiliensis*.

5. Conclusions

LED lamps are a useful tool for applying red light irradiance in post harvest treatments of *Q. brasiliensis* leaves aiming at increased QB-90 yield. LEDs have shown homogeneous and consistent results for saponins induction and, despite their relatively higher cost, they proved advantageous in comparison to cellophane filters.

The use of combined red light and UV-C irradiation has not show synergistic effect on QB-90 induction. Notwithstanding it was possible to observe an increase tendency on the QB-90 accumulation by using 15 minutes of UV-C irradiation followed by 11 hour and 45 minutes of red light irradiation. The red light irradiation interleaved with three 15 minutes pulses of UV-C irradiation, however, showed QB-90 levels identical to the control, possibly due to excess damage. Based on previous results of Yendo et al. (2015) that shown a real improvement of QB-90 content by using whether UV-C for 15 minutes or only

red light irradiance for 12 hours, it seems more advantageous use just one type of light to induce accumulation of QB-90 in detached leaves of *Q. brasilieinsis*.

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ANEXOS

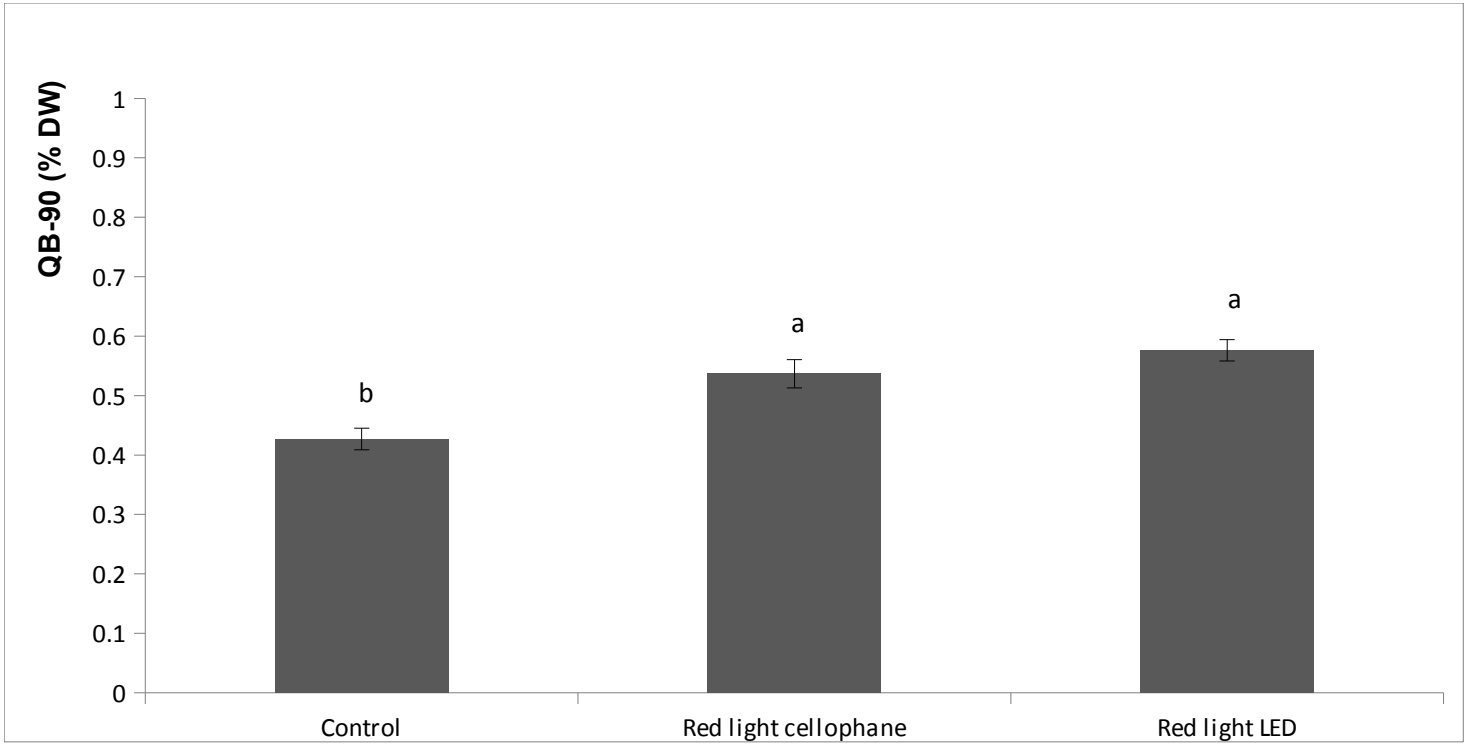

Fig. 1. Concentration of QB-90 saponins in detached leaves of *Quillaja brasiliensis* in untreated (control), treated with 12 hours of red light with cellophane filter and treated with 12 hours of red light from LED lamps. Bars (means ± standards errors) not sharing a letter are significantly different by a Duncan test ($P \leq 0.05$).

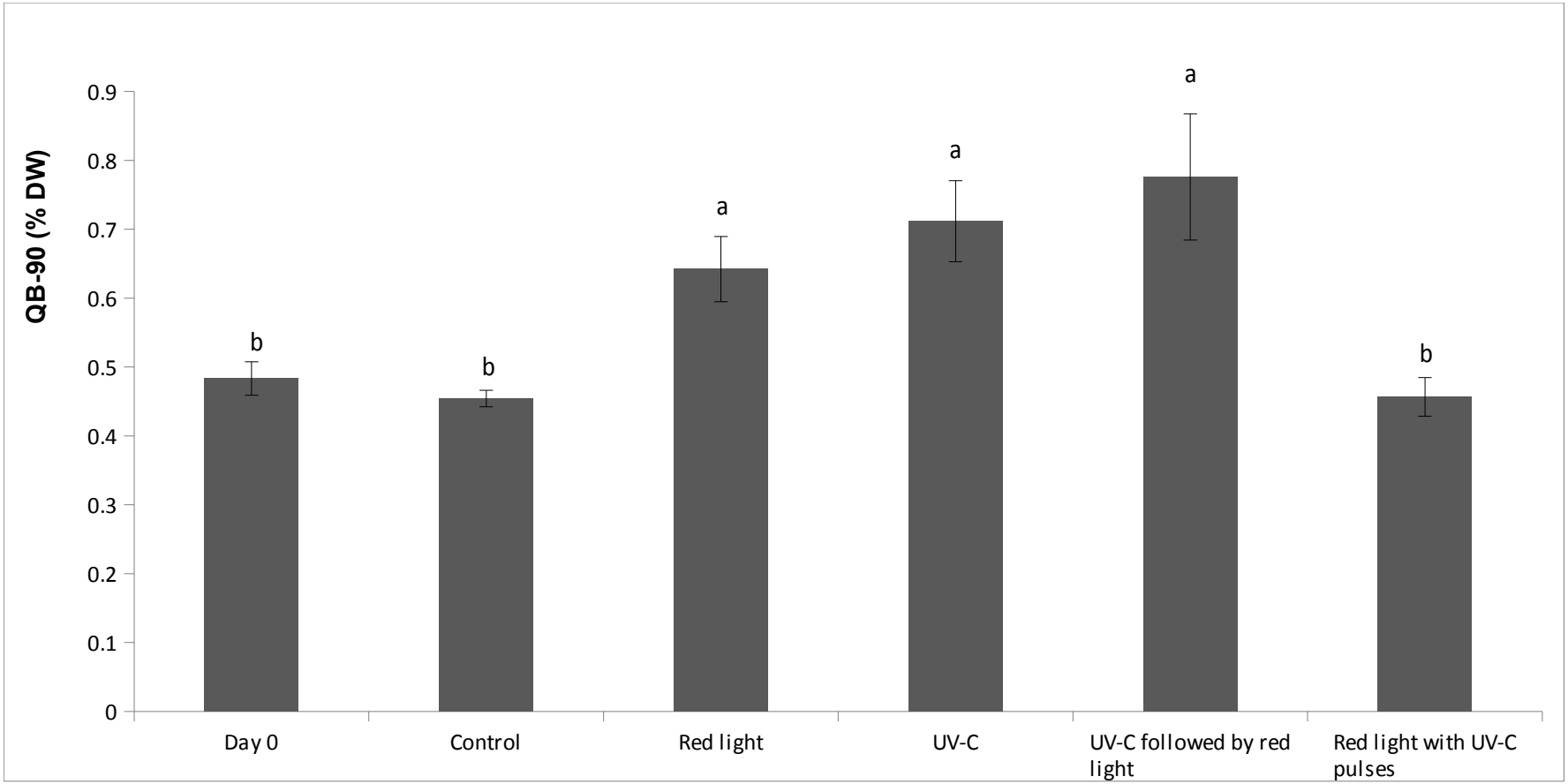

Fig. 2. Concentration of QB-90 saponins in detached leaves of *Quillaja brasiliensis* in basal conditions (day 0), in untreated (control), treated with red light (using LED lamps), treated with 15 minutes of UV-C followed by white light, treated with UV-C followed by red light and treated with red light with 3 pulses of UV-C. All treatments (except day 0) had 12 hours of duration. Bars (means ± standards errors) not sharing a letter are significantly different by a Duncan test ($P \leq 0.05$).