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RESEARCH NOTE

Light and temperature conditions affect bioflavonoid accumulation in callus cultures of *Cyclopia subternata* Vogel (honeybush)

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Abstract Callus cultures of the endemic South-African legume *Cyclopia subternata* were cultivated under varying light and temperature conditions to determine their influence on biomass growth and bioflavonoids accumulation. Experimental modifications of light included complete darkness, light of different spectral quality (white, red, blue and yellow) and ultraviolet C (UVC) irradiation. The calli were also subjected to elevated temperature or cold stress. Among the tested light regimes, cultivation under blue light resulted in the highest levels of hesperidin (H)—118.00 mg 100 g^{-1} dry weight (DW) on 28 days of experiment, as well as isoflavones: 7-*O*-*β*-glucosides of calycosin (CG), pseudobaptigenin (PG) and formononetin (FG)—28.74, 19.26 and 10.32 mg 100 g^{-1} DW, respectively, in 14-days old calli. UVC irradiation applied on 20 days stimulated the accumulation of H (204.14 mg 100 g^{-1} DW), CG (31.84 mg 100 g^{-1} DW) and PG (18.09 mg 100 g^{-1} DW) in 28 days culture by 140, 46 and 165 %, respectively, without negatively influencing callus growth. Low temperature (13 °C) increased CG content by over 1,500 % (235.29 mg 100 g^{-1} DW) when applied during the whole 28-days growth cycle, at the same time causing 95 % decrease in culture growth in comparison to reference calli maintained at 24 °C. On the contrary, elevated temperature (29 °C) applied during the second half of the culture period resulted in over 300 and 500 % increase in CG and PG content (61.76 and 58.89 mg 100 g^{-1}, respectively) while maintaining relatively high biomass yield.

Keywords Hesperidin · In vitro cultures · Isoflavones · Light spectral quality · Temperature regime · UVC irradiation

Abbreviations

CG	Calycosin 7-*O*-*β*-glucoside
4-CPPU	*N*-(2-chloro-4-pyridyl)-*N*′-phenylurea (forchlorfenuron)
DW	Dry weight
FG	Formononetin 7-*O*-*β*-glucoside
Gi	Growth index
H	Hesperidin
MS	Murashige and Skoog
PG	Pseudobaptigenin 7-*O*-*β*-glucoside
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
UVC	Ultraviolet C

The South-African shrubs of the genus *Cyclopia* (Fabaceae) are used to manufacture the traditional, sweet-scented herbal tea, commonly known as honeybush. They contain a range of biologically active polyphenols, including xanthones, benzophenones, flavanones, flavones, dihydrochalcones and isoflavones. Among the last group are the methoxy-substituted derivatives, represented by calycosin and formononetin (Louw et al. 2013). These compounds, and/or their corresponding glucosides, were shown to exhibit multidirectional biological effects

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including estrogenic (Chen et al. 2013; Louw et al. 2013) and antitumor (Chen et al. 2013; Zhang et al. 2013). However, unlike soybean phytoestrogens (i.e. genistein and daidzein), methoxylated isoflavones like calycosin are less available for biological activity studies. Chinese plants of the genus *Astragalus*, considered a major source of free and glucosidated calycosin, are threatened by overexploitation because of slow growth of the roots combined with high market demand for natural medicines (Wu et al. 2011; Xu et al. 2011).

Cell cultures of *Cyclopia subternata* were previously shown to accumulate 7-*O*-*β*-glucosides of calycosin, pseudobaptigenin and formononetin, absent in intact plant material (Kokotkiewicz et al. 2012, 2013), and can thus be utilized for the production of these derivatives independently of their natural resources. In the present work, the effect of varying light and temperature regimes on biomass growth and accumulation of bioflavonoids in *C. subternata* callus cultures (Kokotkiewicz et al. 2009) was investigated.

For each experiment, 1.5 g portions of callus (grown on MS medium containing 3.0 % w/v sucrose, 20.19 μM 4-CPPU, 1.96 μM 2,4,5-T and solidified with 0.7 % w/v agar, taken on 20 days of the growth cycle) were transferred into baby food jars containing 25 ml of the growth medium with the same composition (culture containers and reagents from Sigma-Aldrich, St. Louis, US-MO) and closed with polypropylene caps. The cultures were maintained in complete darkness or under continuous light (88 ± 8 μmol m^{-2} s^{-1}, TLD 35W white fluorescent tubes, Philips, Amsterdam, Netherlands), applied directly or through colour filters (106 primary red, 101 yellow, 119 dark blue, Lee Filters, Andover, UK). For ultraviolet C (UVC) exposure, polypropylene lids of growth vessels were removed in aseptic conditions and the calli were irradiated for 5 min from 0.15 m distance (TUV 30W/G30 T8 lamp, Philips, Amsterdam, the Netherlands). Except for temperature modification experiments, the cultures were grown at 24 ± 1 °C. The specific experimental schemes are given in Figs. 1, 2, 3 and 4.

The calli were harvested after 14 or 28 days and their growth indices calculated (Fig. 1). The samples were freeze-dried, extracted and assessed for the production of isoflavonoids (CG, PG, FG) and flavonoids (H), identified as major phenolic metabolites in the investigated cultures (Kokotkiewicz et al. 2009, 2012), using the previously described HPLC method (Kokotkiewicz et al. 2009, 2013).

Lighting conditions are well-known factors affecting primary and secondary metabolism in plant cell cultures (Ramakrishna and Ravishankar 2011). The current experiments demonstrated that the light regime did not significantly influence the growth of *C. subternata* callus. All biomasses were characterized by fast growth, with Gi values exceeding 1,000 % after the 28-days experiment

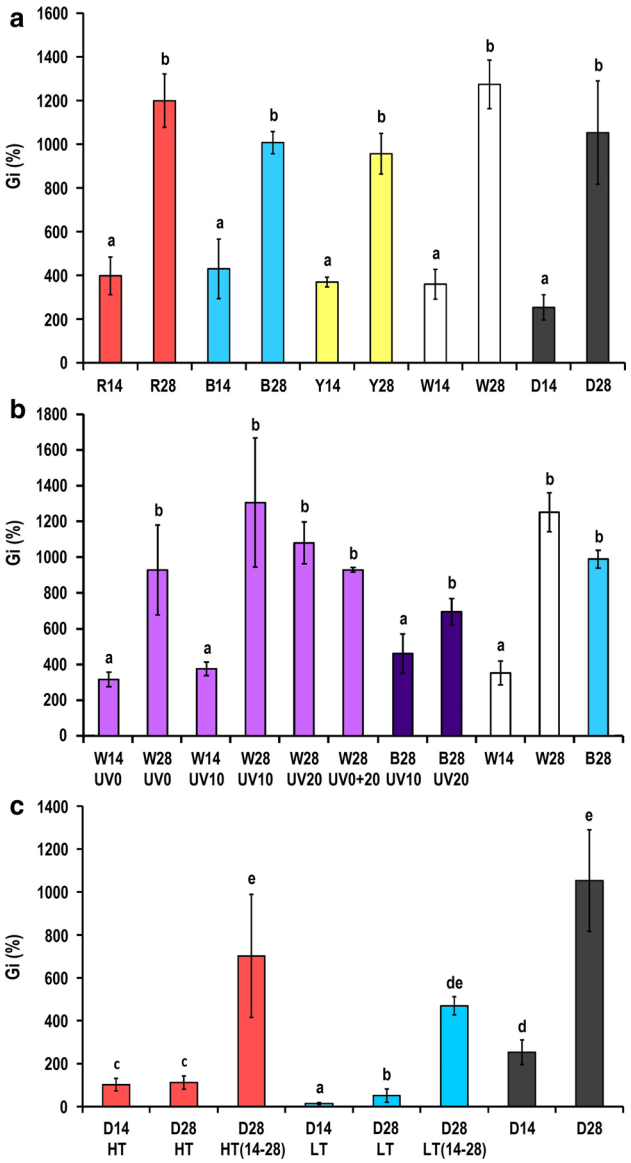

Fig. 1 The effect of different light conditions (**a**), UVC irradiation (**b**) and temperature regimes (**c**) on the growth of *C. subternata* callus. Experimental modifications applied: *R* red light; *B* blue light; *Y* yellow light; *W* white light; *D* darkness; *14, 28* callus grown for 14 or 28 days, respectively; *UV0, UV10, UV20, UV0+20* UVC exposure on 0 (start), 10, 20 or 0 and 20 days (double irradiation) of the experiment, respectively; *HT, LT* experiment maintained at 29 or 13 °C, respectively; *14–28,* elevated/lowered temperature applied only during a second half of the experiment. Growth indices were calculated as follows: $Gi = [(G_1 - G_0)/G_0] \times 100$ % where Gi is the growth index, G_1 is the callus fresh weight at the end of a cultivation period and G_0 is the fresh weight of the inoculum. *Different letters* indicate significant differences between means (n = 3) based on Tukey's range test ($p < 0.05$). (Color figure online)

(Fig. 1a). On the other hand, light conditions proved to significantly affect bioflavonoids accumulation. The presence of light showed to be beneficial, but not necessary, for the production of the examined compounds. The highest amounts of H (28 days) and isoflavones (14 days)

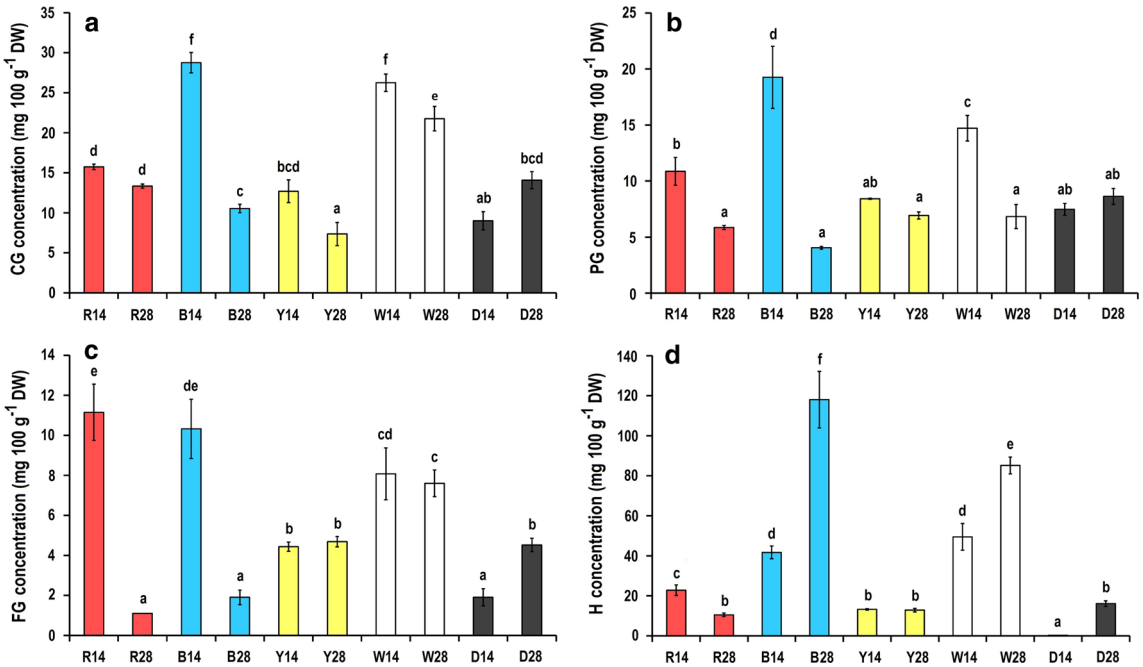

Fig. 2 The effect of different light conditions on the accumulation of calycosin 7-*O*-*β*-glucoside (**a**), pseudobaptigenin 7-*O*-*β*-glucoside (**b**), formononetin 7-*O*-*β*-glucoside (**c**) and hesperidin (**d**), in *C. subternata* callus. Abbreviations and statistical designations as defined in Fig. 1. (Color figure online)

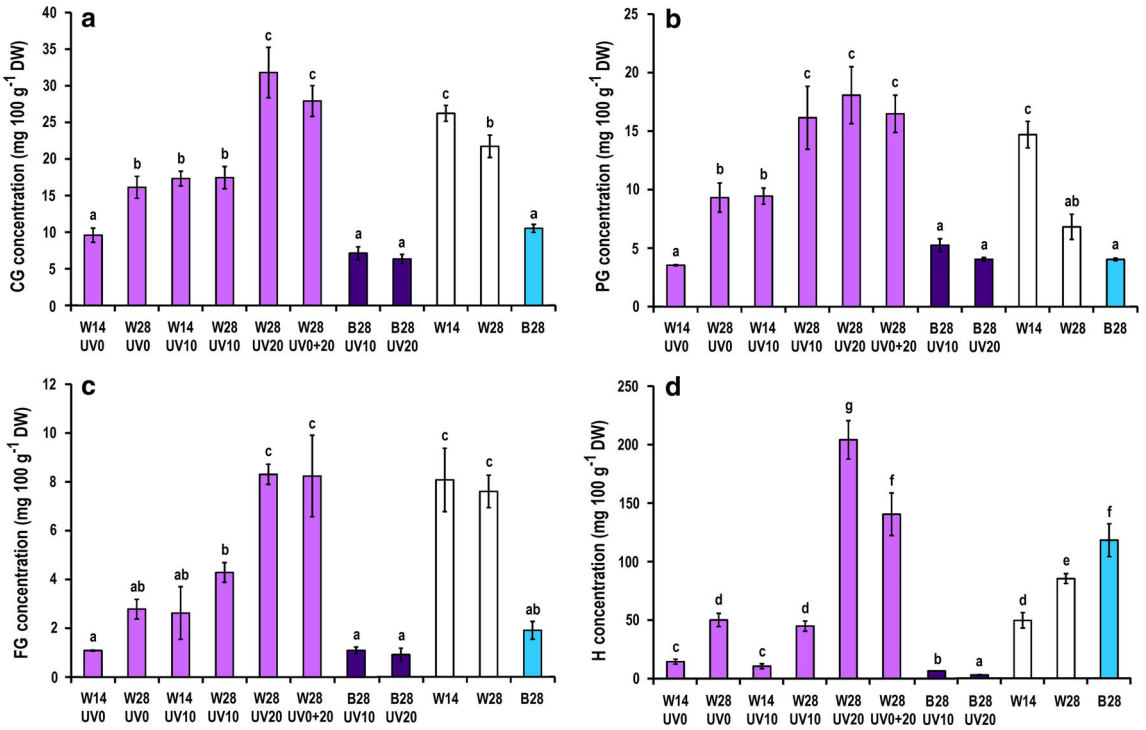

Fig. 3 The effect of UVC irradiation on the accumulation of calycosin 7-*O*-*β*-glucoside (**a**), pseudobaptigenin 7-*O*-*β*-glucoside (**b**), formononetin 7-*O*-*β*-glucoside (**c**) and hesperidin (**d**), in *C. subternata* callus. Abbreviations and statistical designations as defined in Fig. 1. (Color figure online)

were recorded in the calli maintained under blue light (Fig. 2). This is in agreement with previous reports, indicating the stimulatory effect of blue light on the accumulation of plant phenolics in in vitro cultures, with examples including flavonoids in *Saussurea medusa* calli (Guo et al. 2007) and phenolic acids in shoots of *Ruta*

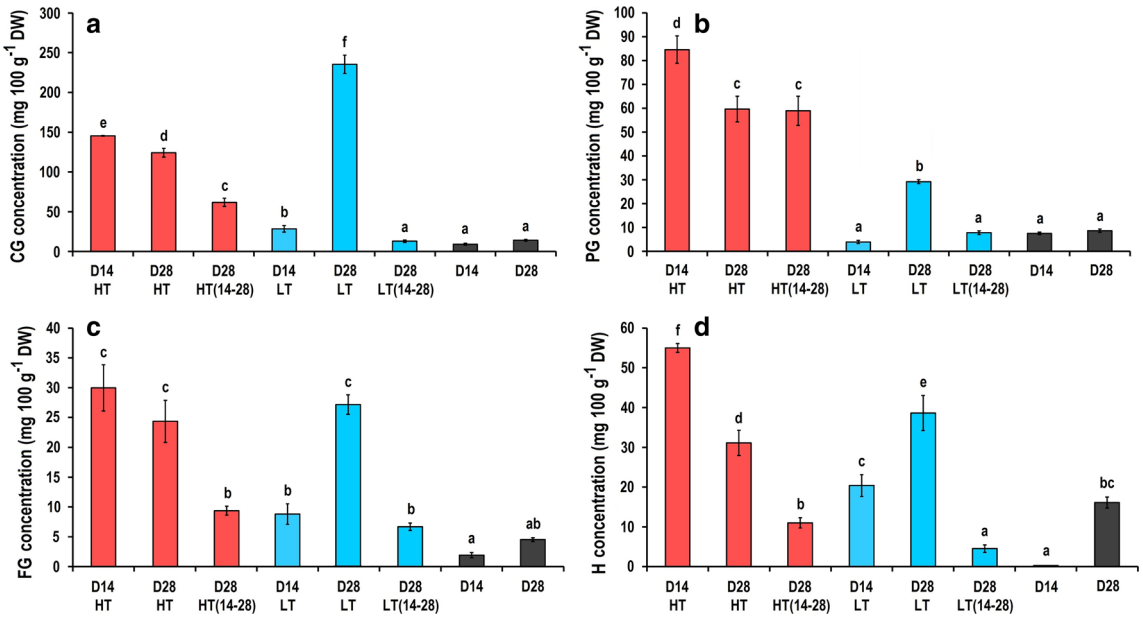

Fig. 4 The effect of different temperature regimes on the accumulation of calycosin 7-*O*-*β*-glucoside (**a**), pseudobaptigenin 7-*O*-*β*-glucoside (**b**), formononetin 7-*O*-*β*-glucoside (**c**) and hesperidin (**d**), in *C. subternata* callus. Abbreviations and statistical designations as defined in Fig. 1. (Color figure online)

graveolens (Szopa et al. 2012). Unfortunately, in the present work the production of the most valuable metabolites (i.e. isoflavones) was not correlated with high biomass yield, thus limiting the usefulness of the above described experimental scheme.

Further part of the study included irradiation of the calli using UV light, which was previously shown to increase the accumulation of isoflavones in legume plants—the examples include genistein in *Genista tinctoria* callus (Tůmová and Tůma 2011) and methoxylated isoflavones (calycosin, formononetin and CG) in *A. membranaceous* leaves (Xu et al. 2011). It was decided to subject *C. subternata* callus to highly energetic UVC radiation, which so far has been scarcely studied with respect to its influence on isoflavone biosynthesis. Due to the high energy of UVC, it can be conveniently used in plant cell culture experiments as a stress factor, applied in short bursts at the desired moments of the growth period.

As presented in Fig. 1b, there were no statistical differences between the Gi values of calli grown without UVC treatment and subjected to various irradiation schemes. To the contrary, the effects of UVC on bioflavonoid accumulation were clearly dependent on the moment of exposure (Fig. 3). Irradiation on 0 or 10 days of the experiment resulted in statistically unchanged (CG and PG) or lowered (FG and H) secondary metabolite concentrations on 28 days, as compared to the control. On the other hand, application of UVC on 20 days significantly increased the levels of CG, PG and H by 46, 165 and 140 %, respectively, in the cultures maintained under white light, yielding the best results in the described series of experiments. As opposed to the experiments depicted in Fig. 2, this strategy also enables to match the high biomass yield with high secondary metabolite accumulation (both achieved on 28 days).

The parallelly conducted experiments involved cultivation of *C. subternata* calli under elevated (29 °C) and lowered (13 °C) temperatures (as compared to standard 24 °C), as both these strategies were effective in increasing isoflavone accumulation in the selected legume species. For instance, Thanonkeo and Panichajakul (2006) reported over twofold increase in biomass growth and threefold higher isoflavone content in *Pueraria candollei* callus grown at 32 °C. On the other hand, cold stress was shown to cause a significant increase in calycosin and its glucoside content in *A. membranaceus* seedlings (Pan et al. 2007).

As shown in Fig. 1c, elevated temperature almost completely inhibited callus growth, which is indicative of its high sensitivity to environmental changes. Thus, despite the significantly increased isoflavone levels (Fig. 4), the above approach has no practical value for the production of the examined compounds. In order to stimulate isoflavone biosynthesis without compromising biomass growth, the calli were subjected to elevated temperature only during the second half of the culture period. This strategy proved partially successful—the relatively high growth rate was maintained but increased accumulation was recorded only for CG and PG (over 300 and 500 %, respectively).

Cold stress was shown to significantly inhibit the growth of *C. subternata* callus. However, low temperature applied during the whole culture period resulted in elevated

bioflavonoid accumulation. The highest (>1,500 %) increase was recorded for CG, thus confirming the results by Pan et al. (2007). Unfortunately, the attempt to correlate isoflavone production with high biomass yield, by applying cold stress only during the 14–28-days period, proved unsuccessful and resulted in secondary metabolite concentrations comparable to the control group (Fig. 4).

Summing up, bioflavonoid accumulation in *C. subternata* callus was shown to be strongly affected by light and temperature regime applied. The best results, in terms of biomass growth and secondary metabolite content, were obtained by maintaining the cultures at 29 °C during the second half of the growth cycle, or by UVC exposure of the callus on 20 days of experiment. These strategies may be useful for biotechnological production of methoxylated isoflavoned based on *C. subternata* cell cultures.

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