Effect of light-emitting diodes on the proliferation of immature endosperm derived calli of coconut (Cocos nucifera L.)

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Research Article

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Abstract

Coconut endosperm has a unique fatty acid profile with a pre-dominance of saturated fatty acids like lauric acid and myristic acid. Manipulation of fatty acid biosynthesis pathways can be possible with in vitro multiplication of endosperm tissue in this important oil yielding perennial palm tree. In this study, influence of Light Emitting Diodes (LEDs)(blue, yellow and purple colored) was investigated on in vitro initiation and proliferation of coconut endosperm calli. Biomass accumulation, total soluble sugars, reducing sugars, free amino acids, total polyphenols and fatty acid profile were estimated from endosperm calli of Gangabondam Green Dwarf (GBGD) cultivar grown under different colored LEDs. Calli grown under dark conditions served as control. Results indicated that initiation of calli was faster under dark conditions, whereas the multiplication and proliferation was significantly high under purple LED. Production of total soluble sugars, reducing sugars, total polyphenols and fat contents were enhanced in cultures under LEDs in comparison to control. Fatty acid profiles, generated through gas chromatography (GC), indicated that medium chain saturated fatty acid content was more, while long chain saturated and unsaturated fatty acid content was less in cultures grown under LEDs compared to cultures under control conditions. It is evident from the results that coconut endosperm calli can be initially initiated under dark conditions and it has the potential to proliferate under LED conditions with significant impact on fatty acid synthesis.

Introduction

One of the key members of monocotyledonous Arecaceae family and the solitary species under the genus *Cocos* (Rivera et al. 1999), the coconut palm(*Cocos nucifera* L.) is cultivated in more than 12.25 million hectares across 89 tropical countries (ICC 2021). This important fruit tree crop is morphologically classified in to cross-pollinating tall and predominantly self-pollinating dwarf cultivars (Rajesh et al. 2018). Even though popularly referred by the sobriquet "Tree of Life" for being a service provider in various sectors to the humanity, the primary product is still its solid endosperm derived edible oil (Abraham and Mathew 1963). During triploid endosperm tissue maturation, a part of coenocytic liquid endosperm accomplishes cellular form and gets accumulated with oil globules and other food reserves(Cutter et al. 1955).

Oil content in mature solid endosperm of coconut ranges from 55–65% and is composed primarily of saturated fatty acids with lauric acid (C12:0) and myristic acid (C14:0) being the major fatty acids (Suryani et al. 2020). Apart from being an integral part in food industry, lauric oils finds myriad applications in the manufacture of soaps, detergents, paints and cosmetics (Kumar 2011). Owing to its unique fatty acid profile, research on coconut endosperm has been gaining interest and led to in vitro culturing of this triploid tissues. Fisher and Tsai (1978), have reported the initiation of callus from endosperm tissues in a dwarf coconut variety 'Malayan Dwarf Orange', but the results were inconsistent. Kumar et al. (1985) reported successful proliferation of endosperm calli from a tall coconut variety ‘West Coast Tall’ even though, naturally endosperm tissue is considered to have limited *in vivo* growth. Different basal media tested for in vitro endosperm culture were Branton and Blake (1986) by

Sukampto(2011), Eeuwen's Y3 (1976) by Kumar et al. (1985) and Schenk and Hildebrandt (1972) by Fisher and Tsai (1978). Majority of these culture media were supplemented with2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) as plant growth regulators (PGR) and activated charcoal (Kumar et al. 1985; Sukampto 2011). Recently transcriptome assembly of coconut endosperm callus and its functional annotation has been reported by Venugopal et al.(2021).

Optimization of culture conditions plays a crucial role in enhancement of in vitro biomass in plant tissue culture endeavors (Isah et al. 2018). Extrinsic factors surrounding the cultures are known to influence growth and development of in vitro cultures. Light irradiance is one of the important external factors which influences the internal environment of the culture vessel containing cultures (Huang and Chen 2005). Prior reports have suggested that initiation and proliferation of endosperm calli is achieved under dark conditions (Kumar et al. 1985; Sukamto 2011).In recent times, light emitting diodes (LED's) are being preferred as light source in in vitro cultures owing to their stability, coolness, low energy consumption and small size (Alvarenga et al. 2015). Report suggests the beneficial effects of LEDs on in vitro tissue regeneration (Zielinska et al. 2020) and callus proliferation (Lian et al. 2019; Saldarriagaet al. 2020). Biosynthesis of secondary metabolites and other active compounds were reported to increase via enhanced signal transduction under LED exposure of in vitro cultures (Kishimaet al. 1995; Juneja et al. 2022). Hence, it would be interesting to understand the influence of different colored LEDs on in vitro growth and development of coconut endosperm cultures in vitro especially with respect to fatty acid profile and biochemical constituents. To the best of our knowledge, this would be the first report on influence of different colored LEDs on proliferation of endosperm calli of coconut.

Materials and methods

Plant materials Seven months (after fertilization) old immature nuts from Gangabondam Green Dwarf (GBGD) cultivar maintained at the Experimental Farm of ICAR-CPCRI, Kasaragod, Kerala State, India (12.30^{o} N, 75^{o} E; altitude of 10.7m above MSL) were taken. The nuts were surface sterilized using alcohol (95%), and after minimal processing by removing the husk in the upper half of the nuts, were taken to a laminar air flow chamber. After flame sterilization, the anterior portion was opened with a knife and liquid endosperm was decanted. A layer of endosperm (~ 1.5 cm diameter), along with a thin layer of the shell (~ 5 mm) at the micropylar region (the portion where the zygotic embryo is located) was then sampled using a surgical blade (HiMedia, India, No. 24) attached to a scalpel holder (HiMedia, India,No. 4).

Media and culture conditions Explants were inoculated in Y3 medium (Eeuwens 1976) supplemented with sucrose(30 g L^{-1}), 2,4-D (18.1 µM), NAA (21.5 µM), BAP (2.2 µM), agar (7 g L^{-1}) and activated charcoal (2 g L^{-1}) (Venugopal et al. 2021). The pH of the medium was adjusted to 5.75 before autoclaving the medium. In the first experiment, cultures were incubated under different colored LEDs, *viz*., yellow (Y-LED), blue (B-LED) and purple (equal Red and Blue; P-LED) while cultures in dark served as control. Different colored LED strips available locally were installed on the trolleys to obtain a light

intensity of ~ 20 µmol m^{-2} s^{-1} measured through a Photosynthetically Active Radiation (PAR) sensor connected with LiCOR 6400 XT (USA), and the photoperiod was regulated to 16hrs a day with a digital timer. Cultures including the control were maintained at 27 ± 1ºC. Sub-culturing was done in monthly intervals to the same basal medium with half the concentrations of growth regulators.

In the second experiment, cultures were incubated in dark during callus initiation. After 12 weeks, the calli (1.5-2 g Petri $plate^{-1}$) in multiplication medium with2,4-D (0.5µM) alone was subjected to different LED treatments to understand the effect on proliferation of calli.

Effect of LEDs on proliferation, total soluble sugars, reducing sugars, total polyphenols, total free amino acids and fat content in coconut endosperm calli Accumulation of callus biomass was quantified at monthly intervals and relative growth rate was calculated using the formula RGR = $\frac{(lnW2 - lnW1)}{(T2 - T1)}$ where W2 and W1 are fresh weight of callus at T2 and T1 time interval, respectively. After four months, 100 g of calli from each treatment was kept at 60ºC for 48 hrs to reduce the moisture content by 90% and fat was extracted using a Soxhlet apparatus by placing the desiccated calli in cellulose thimble and adding 85 mL of petroleum ether (40-60^{o}C boiling range). Extraction was carried out for 90 minutes and petroleum ether with fat was kept in a pre-weighed glass bowel. After complete evaporation of petroleum ether by keeping in an oven at 60^{o}C, they were weighed again and fat content was estimated gravimetrically. Defatted callus sample (0.5 g) was ground into powder in a mortar and pestle after adding 10 mL of 80% ethanol. Resulting mixture was kept in a water bath at 70^{o}C for 30 minutes. They were then filtered using Whatman No. 1 filter paper and extracted again using 5 mL of hot 80% ethanol. Filtrate in a boiling tube was kept in water bath at 80^{o}C to evaporate the ethanol. Remaining filtrate was diluted with distilled water to make the final volume to 10 mL and they were vortexed thoroughly. Concentration of total soluble sugars was estimated by following the phenol sulphuric acid method (Dubois et al. 1956), reducing sugar was estimated by following the method of Nelson and Somogyi (Somogyi 1952), total phenols by Folin-Ciocalteu method (Bray and Thorpe 1954) and total free amino acids by the method of Moor and Stain (1948).

Gas chromatography analysis of fatty acid profile on the effect of different LEDs in coconut endosperm calli Methyl esters of fatty acids were prepared for the analysis of fatty acid profile following the method of Padua-Resurreccion and Benzon (1979) and Kumar (2007). The HCl reagent (5%) was prepared by adding 8.3 mL of acetyl chloride drop wise to 100 mL methanol. To prevent bumping owing to exothermal reaction an ice jacket was used. The HCl reagent (2 mL) was added to 50 mg fat, in a 15 mL screw-capped glass vial and vortexed. This mixture was incubated in a hot air oven for 10 h at 70˚C and cooled to room temperature. Distilled water (5 mL) and 1 mL hexane (MERCK) were added to this incubated mixture and vortexed thoroughly to separate two layers. The top hexane layer was carefully pipetted out and stored in microtubes for fatty acid profiling. During the analysis, samples were diluted (40 µL FAME sample + 960 µL n-hexane, MERCK, high performance liquid chromatograph [HPLC] grade)

in the sample vial. Through an auto injector (AOC20i, Shimadzu) connected to Gas Chromatograph (GC-2010, Shimadzu, Kyoto, Japan), sample (1 µL) was injected into capillary column (BPX 70, SGE Analytical Science, Austin, TX). Using a Flame Ionization Detector (Shimadzu), the elutants were detected. The conditions set for analysis were explained elsewhere (Kumar 2007). The injection mode was in split (split ratio 1:50); terminal temperature was 225˚C; nitrogen and air were carrier gases; pressure was set to 114.9 kPa; total flow was maintained at 68.9 mL min^{-1}; and column initial temperature was 100˚C with temperature increase rate of 5˚C min^{-1}. Through GC-Solutions software (Shimadzu), amplified signals were transferred and recorded in a computer. Under similar conditions of analysis, fatty acid methyl ester standards (C6-C24) procured from Sigma-Aldrich, Supelco, Bellefonte, PA, were run before the actual sample analysis.

Statistical analysis All the data were represented as mean ± standard error of the mean. Values from the experiment pertaining to each trait were compared using one-way ANOVA and DMRT post hoc analysis in SPSS V 17. All data are representative of three replications (n = 3 error bars, SEM).

Results

Effect of LEDs on induction of endosperm calli Results indicated that callus induction was faster under dark incubation in comparison with cultures grown under LED. Under complete darkness, induction of calli was evident from the second week post inoculation whereas it took more than four weeks under LEDs. Gain in the fresh weight of callus was five-fold higher in explants under dark condition in comparison with the LEDs. Among the LEDs, gain in callus weight was more in P-LED and lowest under Y-LED (Fig. 1A). Significantly higher relative growth rate in callus weight was noticed in explants grown under dark condition in comparison with the LED treatments. Among the LED treatments lowest growth rate was seen in Y-LED where as it was highest under P-LED (Fig. 1B).

Effect of LEDs on proliferation of endosperm calli Callus proliferation was significantly more in P-LED and it was lowest in B-LED (Fig. 2A). Results indicated that after four months of treatment, increase in weight of calli in purple LED was to the tune of 35% in comparison with the cultures in dark. The increment in callus weight under Y-LED was not significant. Faster proliferation under P-LED was reflected with the significantly higher relative growth rate (RGR) in the fresh weight of callus of 0.042 g g^{-1} day^{-1} whereas it was 0.034 g g^{-1} day^{-1} in control. The RGR was lowest in B-LED and the difference under Y-LED was not significant (Fig. 2B).

Effect of LEDs on biochemical traits of endosperm calli Results indicated that, in general, the concentration of total soluble sugars, reducing sugars and total polyphenols was more in cultures under LEDs in comparison with the cultures grown under dark conditions, whereas the concentration of free amino acids was less in the former treatments. Among the LED treatments, higher concentrations of total soluble sugar and reducing sugar content; and lower concentrations of free amino acids were documented for cultures exposed to P-LED. Total polyphenols and total free amino acid contents were more in B-LED and Y-LED, respectively (Fig. 3).

Fat content in general was more in cultures under LEDs than those grown under dark condition. It was found to be significantly high in Y-LED followed by cultures maintained under B-LED and P-LED (Fig. 4). The increase in the fat content in Y-LED was to the tune of 1.8 times in comparison with the control. The fatty acid analysis indicates that endosperm calli has caprylic acid (RT = 2.83), capric acid (RT = 4.48), lauric acid (RT = 6.9), myristic acid (RT = 9.8), palmitic acid (RT = 12.7), stearic acid (RT = 15.4), oleic acid (RT = 16.0) and linoleic acids (RT = 16.7) as the major fatty acids and represented (Fig. 5). In general, the content of lauric acid, palmitic acid and linoleic acids were more in medium chain saturated fatty acid (MCSFA), long chain saturated fatty acid (LCSFA) and long chain unsaturated fatty acid (LCUFA) categories, respectively. Compared to control maintained under dark conditions, the content of MCSFA such as caprylic acid, capric acid and lauric acid were more in cultures grown under LEDs. Among these, increment in lauric acid was more in comparison with the other two fatty acids. Among LEDs, the MCSFA content was highest in Y-LED and lowest in P-LED (Fig. 5A &B). With the exception of myristic acid, which followed the similar trend as MCSFA, other two LCSFA such as palmitic acid and stearic acid content was highest in cultures grown under dark in comparison with those under LEDs. Among LEDs, the content of LCSFA was more in cultures under B-LED (Fig. 5C). Results also revealed that LCUFA content was low in LEDs as both oleic acid and linoleic acid content was significantly high in control under dark condition. In LED treatments, content of these two fatty acids were lowest in Y-LED and highest in P-LED (Fig. 5D).

Discussion

Coconut, possessing a high amount of medium chain fatty acids in its solid endosperm, is an economically important palm grown around the tropics. Production of bioactive compounds are possible via callus cultures and are affected by several external factors such as light (Cardoso et al. 2019). Even though efforts have been made since the 1980s toward studying in vitro culturing of solid coconut endosperm (Kumar et al. 1985), information on the influence of external factors such as light on induction and proliferation of endosperm calli are not available for this recalcitrant species. In this study, we have compared the initiation, multiplication and biochemical composition of calli derived from immature endosperm explant of coconut under complete darkness with 16 h different LED light/8 h dark photoperiod.

Results of the present study revealed that induction of calli was quick under complete darkness with significant accumulation of biomass over the period of time in comparison with explant in LEDs. To overcome the phenolic exudation from the explants, activated charcoal at the rate of 2 g L^{-1} was supplemented to Y3 medium in the present study. Slow induction of callus under light condition might be due to accumulation of phenolic substances since layer of immature endosperm from micropylar region along with a thin layer of shell formed the explant. Induction of callus is restricted to dark condition in variety of explants from several plant species (Yusnita and Hapsoro 2011; Deng et al. 2020; Suhartanto et al. 2022) as light may affect callus development by alteration in endogenous auxin and gibberellins levels (Halliday et al. 2009). It is worth noting that, in spite of slow induction of callus, proliferation and

multiplication was faster under LED conditions. Post induction of callus, friable tissues separated from the thin shell portion were transferred to new medium and allowed for multiplication. Blue, yellow and combination of blue and red resulting in purple colored LED has been reported to increase the biomass of calli in *Vigna aconitifolia*, Cymbidium Orchid and *Panax vietnamensis* (Soni and Swarnkar 1996; Huan and Tanaka 2004; Nhut et al. 2015)

Accumulation of phenolics, an abundant source of antioxidants in tissues is governed by several factors and light is one among them (Kapoor et al. 2018). Reports suggest enhanced phenolic content in callus grown under LED conditions especially in blue light (Lian et al. 2019). The data from the present study suggest the accumulation of phenolics in endosperm calli proliferated under B-LED. Studies have documented that syntheses of the natural products within the in vitro system were affected by several factors. Accumulation of fat in endosperm callus as indicated in the present study affected by the growing conditions under LED. Light, temperature, relative humidity, external and internal growing condition in culture vessel influenced the synthesis of fatty acids in in vitro cultures (Cardoso et al. 2019). Significant difference in fat content in callus under LED condition as observed in the present study driven mainly by increment in lauric acid content, an important MCSFA and corresponding reduction in LCSFA and LCUFA content. On an average LCUFA content reduced under LED condition to higher extent (~ 29%) followed by LCSFA (~ 1%) and increase in MCSFA was 14% (Fig. 6).

It has been reported that raw materials with lower values of LCUFA especially linolenic acid content, would serve more stability to the biofuel (Bocianowski et al. 2012). Coconut in general has higher per cent of MCSFA and reduced concentration of LCUFA towards maturity (Kumar and Balakrishna 2009) which would make it a strong candidate in terms of biofuel production as well apart from food supplement. Hence, large scale multiplication of endosperm can serve as raw material for the generation of biofuel. Present study highlighted the efficacy of P-LED to induce the proliferation of callus and Y-LED and B-LED on synthesis of fatty acids.

Conclusion

It is concluded from the present study that significantly higher proliferation of endosperm calli of coconut can be achieved under P-LED after a callus induction phase under dark condition. Apart from achieving biomass, yellow and blue LED conditions resulted in reasonably high content of fat, polyphenols and MCSFA content.

Declarations

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Conflict of interest The authors declare that there is no conflict of interest.

Author contribution Conceptualization, monitoring and resources: R.M.K. Data curation: G.V., M.K.S. and S.P. Methodology: G.V., M.K.S., S.P. and R.M.K. Formal analysis: G.V., M.K.S. and R.M.K. Writing-original draft: G.V. Writing-review and editing: G.V., M.K.S. and R.M.K.

Data availability All data generated during the study are provided in this manuscript.

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Figures

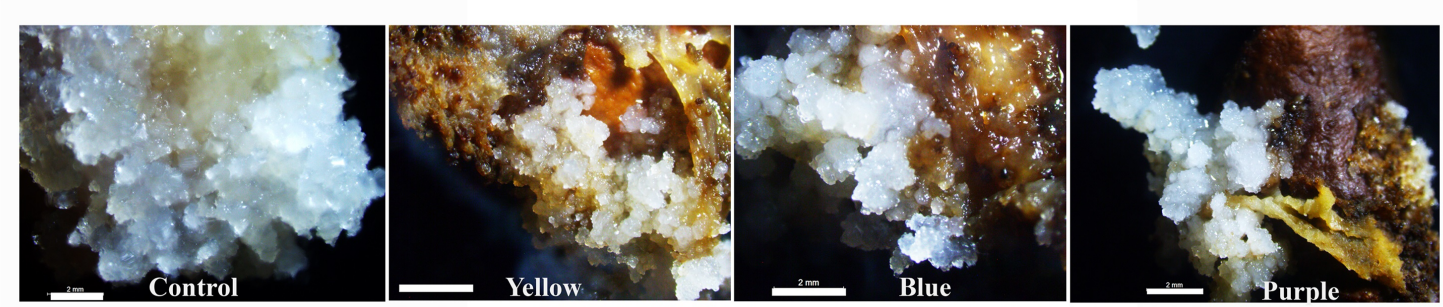

Fig1A

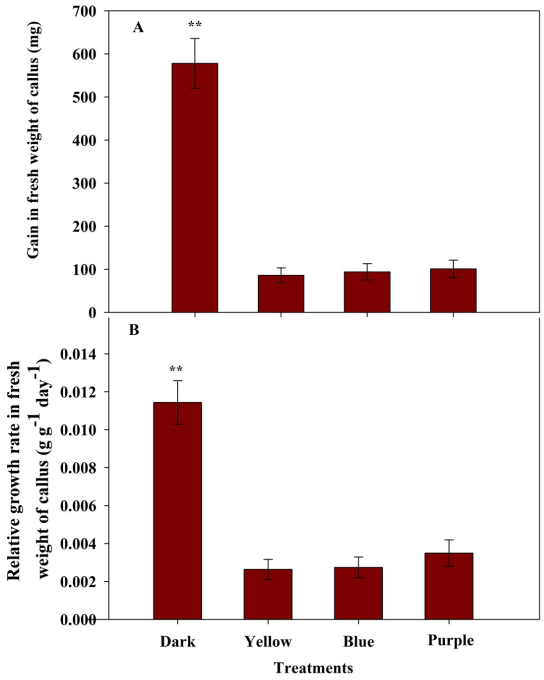

fig1B

Figure 1

Initiation of endosperm calli under different light conditions in immature endosperm cultures of GBGD cultivar. Explants were incubated in dark and in different LEDs for a period of eight weeks and gain in fresh weight of the explant (A) and relative growth rate (B) is represented with standard error of mean. Mean values under different treatments were compared and ** indicated the significance according to DMRT at $P < 0.01$. Representative stereozoom microscopic images of each treatment are with a scale of 2 mm each.

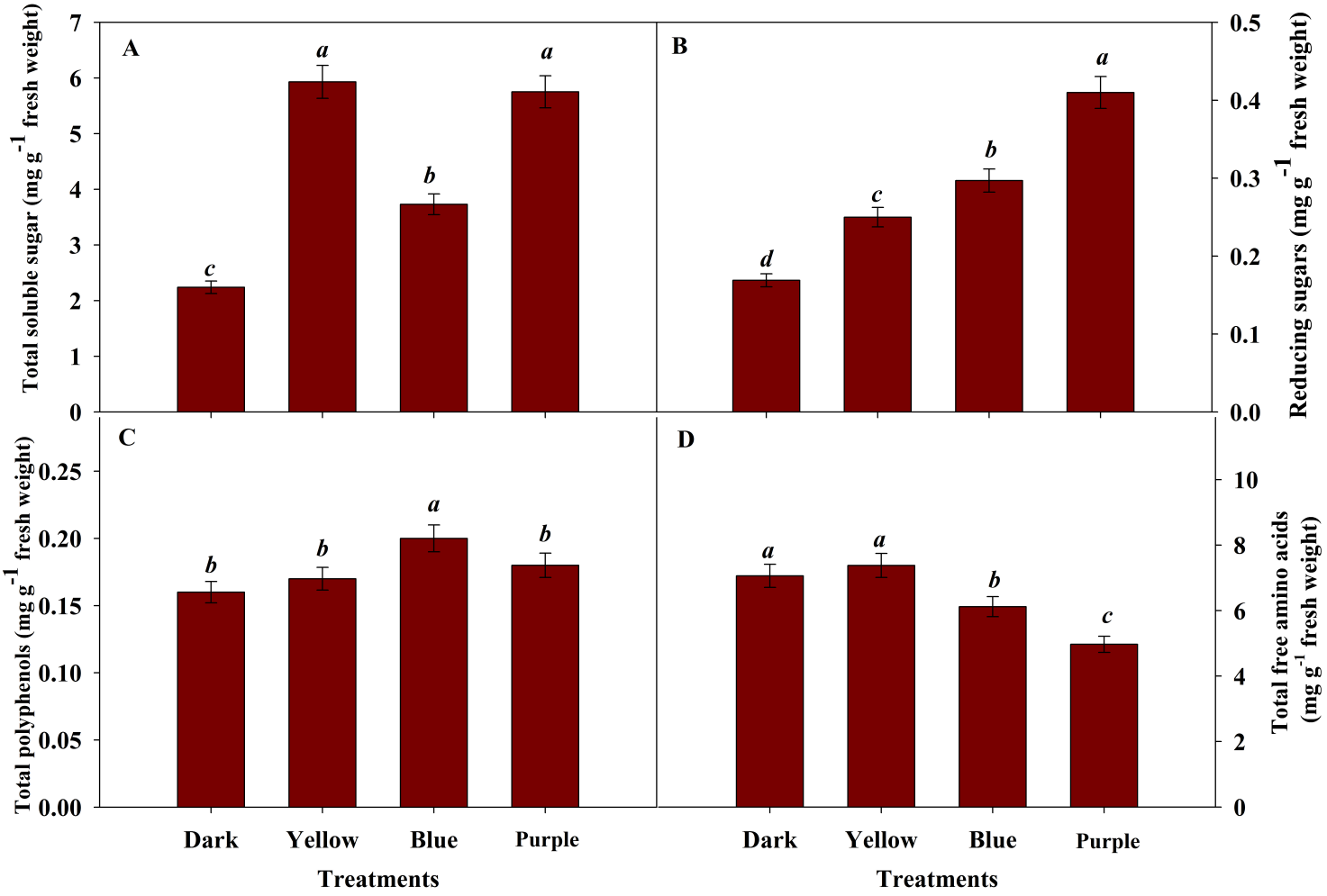

Figure 2

Proliferation of endosperm calli derived from GBGD cultivar and exposed to different LEDs during multiplication stage. Gain in fresh weight of callus four months post treatment (A) and mean relative growth rate in fresh weight (B). Data (n=3) is represented with standard error of mean and different alphabets among the treatments differ significantly according to DMRT ($P < 0.05$).

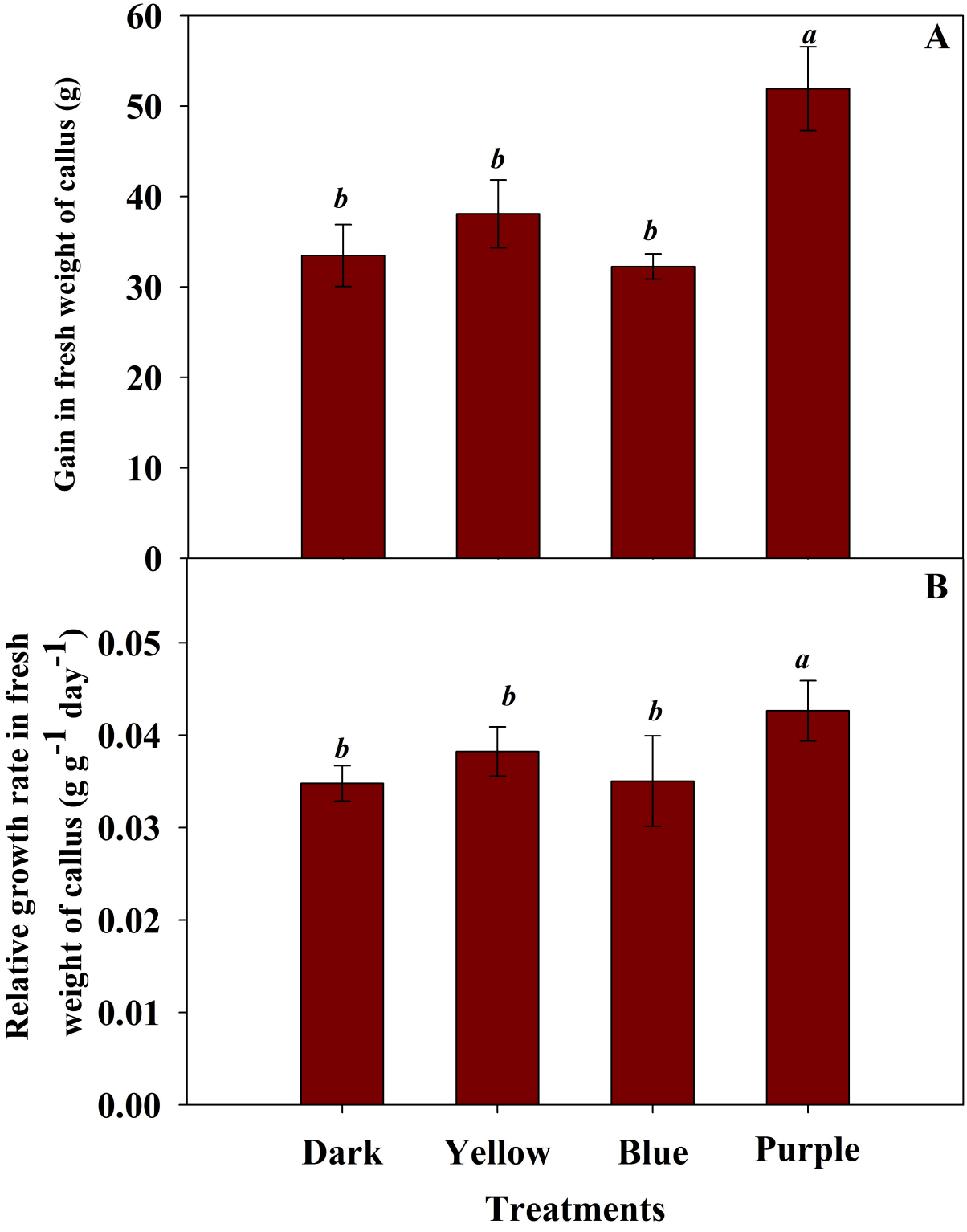

Figure 3

Biochemical traits in endosperm calli of GBGD coconut cultivar exposed to different LED's during multiplication stage. Total soluble sugars (A); reducing sugars (B); total phenols (C) and total free amino acids (D). Mean (n=3) is represented with standard error of mean and different alphabets among the treatments differ significantly according to DMRT ($P < 0.05$).

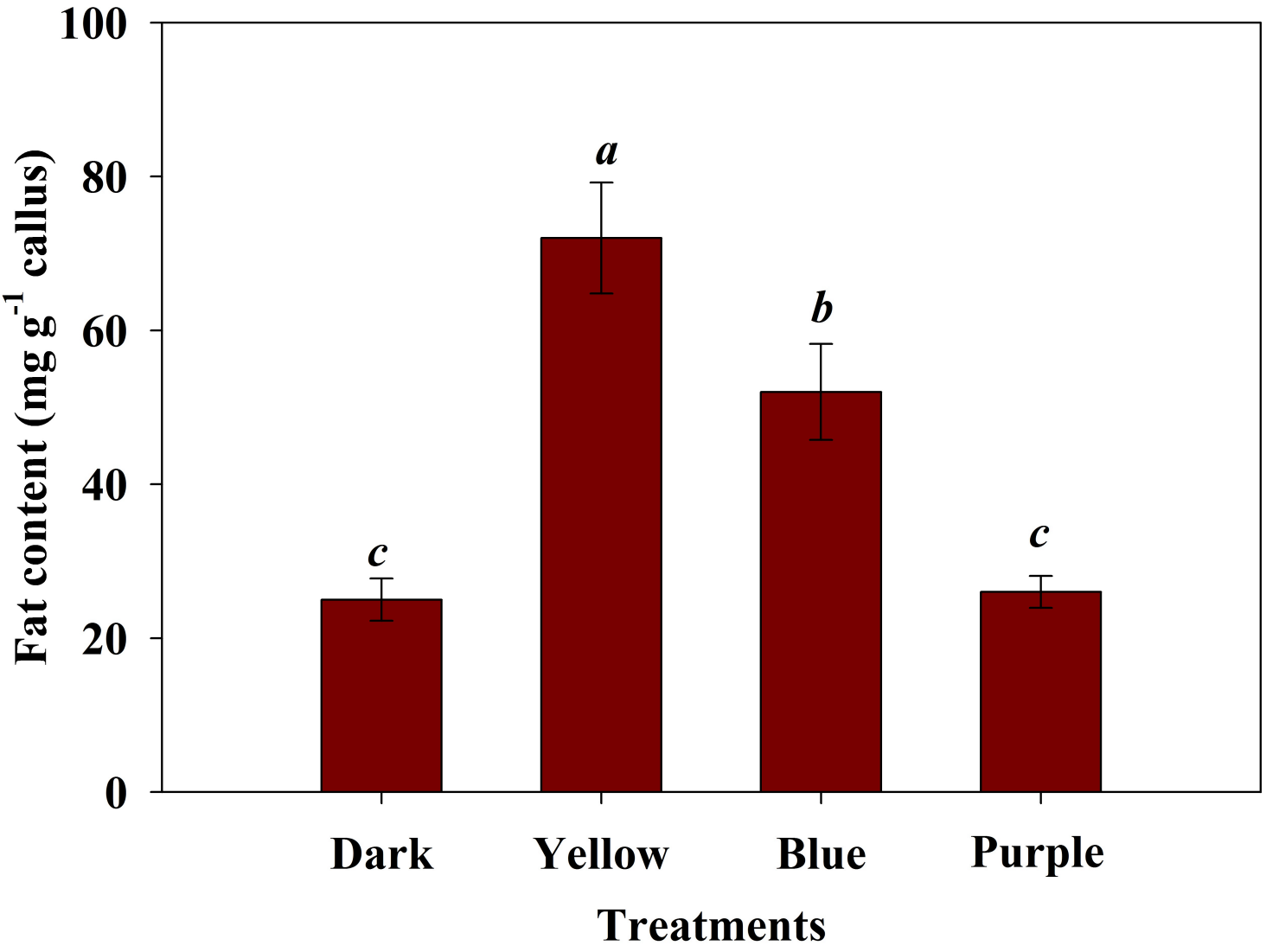

Figure 4

Fat content in endosperm calli of GBGD cultivar exposed to different LED's during multiplication stage. Mean (n=3) is represented with standard error of mean and different alphabets among the treatments differ significantly according to DMRT ($P < 0.05$).

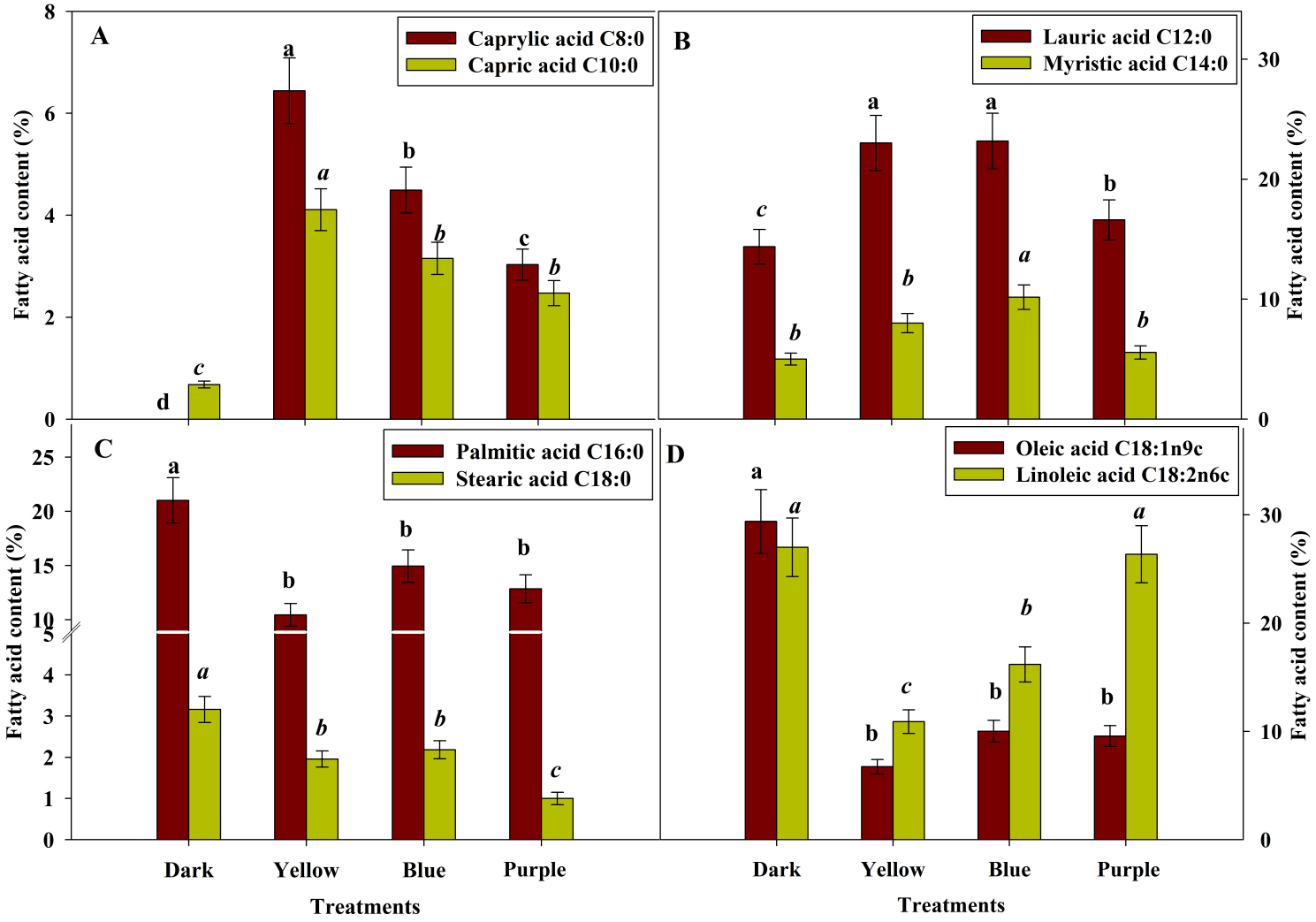

Figure 5

Major fatty acids in endosperm calli of GBGD coconut cultivar exposed to different LEDs during multiplication stage. Mean (n=3) is represented with standard error of mean and different alphabets among the treatments for individual fatty acids differ significantly according to DMRT ($P < 0.05$).

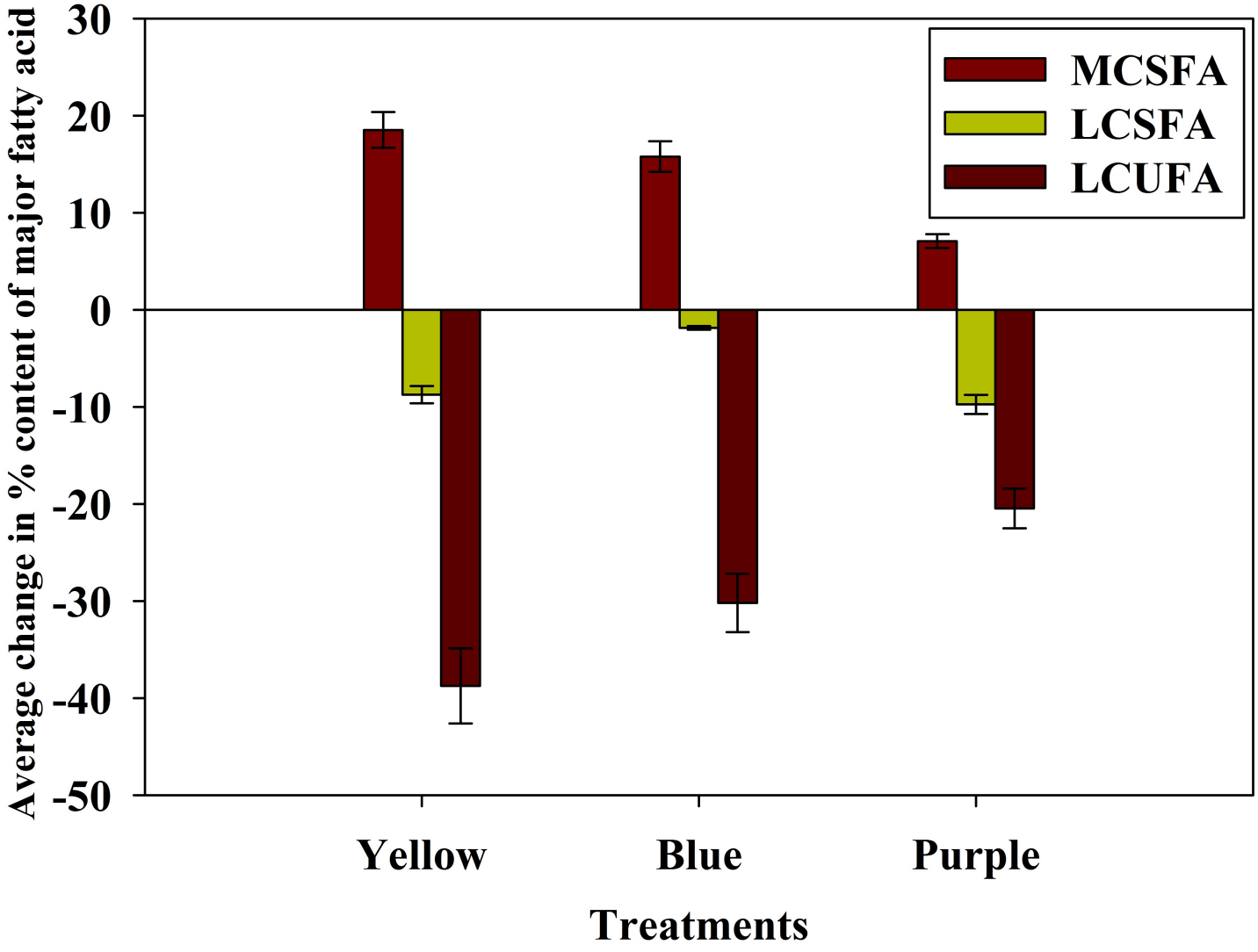

Figure 6

Change in major fatty acid under LED condition in coconut endosperm calli. Average change in MCSFA, LCSFA and LCUFA content under LED condition was compared by subtracting the content of corresponding fatty acids in dark incubation.