Induction of callus culture through plant growth regulators supplementation and the effect of elicitors on enhancement of betalain synthesis using Gomphrena globosa

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Research Article

Keywords: Gomphrena globosa, callus culture, plant pigments, betalain, elicitors, plant growth regulators

Posted Date: June 15th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3050575/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on December 13th, 2023. See the published version at https://doi.org/10.1007/s11240-023-02628-x.

Abstract

Betalains are water soluble nitrogenous pigments produce by plants under the Caryophyllales order and has been favoured as a natural colourant in food and pharmaceutical industries due to its high stability towards pH and temperature over a wide range of food. There is a constant search for alternative source and technique for betalain production to meet the growing demand as conventional extraction method requires high quantity of plant material. Thus, this study sought to examine the potential of producing betalain through callus culture of a natural betalain bearing plant, *Gomphrena globosa* using different plant growth regulators (PGR) and to evaluate the effect of elicitation in enhancing betalain production. Callus induction from different explants showed that the percentage of callus induction (84.00-100.00%) from the leaf and hypocotyl explants was significantly higher than seeds (53.33%). A combination of 0.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) with 1.0 mg/L 6-benzylaminopurine (BAP) were found to be effective inducing pink callus in full strength MS medium. Elicitation with tyrosine was the most effective in enhancing the betacyanin content (red-violet pigments) followed by salicylic acid. The highest betacyanin content, 0.139 ± 0.035 mg/mg FW callus was obtained when 100 µM of tyrosine was supplied. Copper sulphate was found to be effective in increasing the callus size but not the betalain content. The callus size was about 13-fold bigger in MS medium supplemented with 25 µM copper sulphate compared to medium without elicitors. This is the first study reporting an optimised protocol in the production of pigmented callus containing betalain from *G. globosa* using a combination of PGRs consisting of 2,4-D and BAP. In addition, tyrosine can be used as a suitable elicitor to enhance betalain production which provides an alternative source of betalain for the commercial production of natural colorants.

Introduction

Colour is a character for sensory stimulation to influence the perception of flavour (Solymosi et al., 2015). Various food colourings are frequently added in eatables to enhance visual attractiveness or for compensation of natural colour variations that are lost through processing under different harsh conditions (Oplatowska-Stachowiak & Elliott, 2017; Bora et al., 2019). The addition of colour additives is favoured in food industry to enhance the food's pleasantness, palatability, and acceptability towards consumers (Varelis et al., 2018). A colour additive can be further categorized into natural, synthetic, inorganic or natural- identical based on their origins (Fadzliana et al., 2017). Synthetic colourants have quickly gained popularity as they can be produced with lower cost and have higher colour stability (Sigurdson et al., 2017). However, many synthetic colourants such as tartrazine, brilliant blue, carmoisine and erythrosine were later found to be harmful for human consumption (Fadzliana et al., 2017). The growth of fear by consumer for the use of synthetic pigment diverted attention towards naturally derived pigments as a safer alternative. The global market value for natural food colouring ingredients has expanded over the years and is projected to expand further by an annual growth rate of 7.79% by the year 2022 (Novais et al., 2022).

Pigments derived from natural origin have been widely studied and used as colouring agents in foods, cosmetics, and drugs for over generations. Plants are great sources of obtaining natural pigments as they are produced naturally for pollination, and to provide protection for plants against light damage and phytopathogens (Brockington et al., 2011). In the early days, some of the plants that are being used to obtain colour include saffron, paprika, turmeric and different types of flowers (Burrows, 2009; Sigurdson et al., 2017). Chlorophyll, anthocyanins, carotenoids and betalains are the major classes of plant pigments being approved and regulated for industrial use (Scotter, 2011; Solymosi et al., 2015). Among them, betalains are plant derived, water soluble, nitrogen-consisting pigments found only in plants from the Caryophyllales order (Gengatharan et al., 2015; Gandía-Herrero et al., 2016). They can be further classified into two different groups, betacyanin (red-violet) and betaxanthins (yellow- orange). Both compounds are composed of betalamic acid as its core structure (Rahimi et al., 2019; Matencio et al., 2021). Betalains are extensively used in dietary supplements and as food colourants since it is stable at a wide range of pH of 3 to 7 making them as a preferable colour additive for low acid foods (Stintzing & Carle, 2004; Sigurdson et al., 2017; Miguel, 2018). So far, betalain produced from beetroots is a well-established red food colourant that has been used for dairy products such as yogurt, ice cream, frostings, candies, soft drinks, meat substitutes and gelatine desserts (Delgado-Vargas et al., 2010; Khan, 2016).

Despite the promising advantages, betalains are less studied as compared to other pigments due to its limited occurrence in plants (Azeredo, 2009; Polturak et al., 2016). Most betalain-producing plants are restricted under the order of Caryophyllales (Brokington et al, 2011; Timoneda et al., 2019). Beetroot remains as the major and only commercialised source for betalains (Fadzliana et al., 2017; Polturak & Aharoni et al., 2017). Having said that, the betalains derived from beetroot carry an unfavourable earthy smell due to high nitrate concentration (Lu et al., 2003; Fadzliana et al., 2017). Moreover, the betacyanins profile of beetroot has a very high percentage of betanin which limits the colour range produced (Polturak & Aharoni, 2017). The growing condition of beetroots under the soil surface also possesses a risk of contamination by soil-bound bacteria (Stintzing & Carle, 2007; Polturak & Aharoni, 2017). Other plants such as red pitaya, chinese spinach and quinoa have been used to obtain betalain (Biswas et al., 2013; Fadzliana et al., 2017). Utilizing these crops for betalain production is not desirable as they are mainly cultivated for food production and are unable to meet the demand for pigment production (Henarejos-Escudero et al., 2018; Wee et al., 2018). Furthermore, it could be tedious to cultivate the betalain producing plants for betalain extraction in large-scale as the cultivation of plant is seasonal and region dependent (Warhade & Badere, 2018). Hence, the constant search of alternative source is always on the rise.

Utilizing plant tissue culture as a tool, the induction and mass production of callus cultures as a bio-factory to produce pigments could be an alternative approach to the conventional extraction method which requires a large quantity of samples that is not always readily available (de Aguiar Lage et al., 2015). It ensures a consistent and continuous production without compromising the quality and yield of betalain. In addition, the production is not dependent on the climate, soil properties, diseases and pests as the cultures are produced in an aseptic and controlled environment. (Castellanos-Santiago & Yahia, 2008; Miguel, 2018).

A combination of different plant growth regulators (PGRs) and elicitors are often supplemented into the growing media to induce callus formation and to increase secondary metabolites production, respectively. Elicitors can be divided into two groups namely biotic and abiotic. Abiotic elicitors are non-biological origin and are mainly inorganic compounds (salicylic acid, calcium chloride, cobalt chloride and jasmonate) whereas biotic elicitors are biological origin and compounds of cells walls from plants or microbes (chitin, polysaccharides, and yeast extract) (Ramirez-Estrada et al., 2016; Khan et al., 2019). Some of the common abiotic elicitors used to induce pigment production include methyl jasmonate, salicylic acid and copper sulphate (Patel & Krishnamurthy, 2013). Studies have indicated that the supplementation of elicitors can increase the pigment production in various plant species by up to 2-100% but it is highly species dependent (Cai et al., 2012; Saini et al., 2014; Wee et al., 2018). A study conducted by Lakhotia et al. (2014) showed that the 0.5 µM methyl jasmonate increased the synthesis of betalains in *Bougainvillea* spp. by 81.25%. Besides that, Wee et al. (2018) reported that supplementation of 100 µM salicylic acid to callus culture of red pitaya increased the betalains production by two-folds.

This study focuses on the potential of a natural pigment producing species, *Gomphrena globosa* or commonly known as globe amaranth for betalain production (Miguel, 2018). *G.* globosa, under the Caryophyllales order has a high adaptation capacity allowing it to grow and survive in colder regions or poor soil conditions (Silva et al., 2012). Since it is hardy, non-seasonal and a non-food crop, the source of explants for callus induction will be conveniently available. To date, no studies were conducted to initiate callus culture from *G. globosa*. The potential of utilizing callus cultures of *G. globosa* for betalain production remains largely unknown. This is the first comprehensive study that sought to optimise the callus induction of *G. globosa* from the leaf, hypocotyl and seed explants using different combinations of PGRs and to examine the effects of various elicitors (tyrosine, copper sulphate, and salicylic acid) in enhancing the betalain production in the *G.globosa* callus cultures.

Materials and Method

2.1 Plant material and medium preparation

Commercial seeds of *G. globosa* were purchased from JC Garden Seed (product number F122) and used in this study. *In vitro* germination of the *G. globosa* were performed as described by Bodhipadma et al. (2017) with modifications to obtain the leaf and hypocotyl explants. The seeds were first washed with detergent and rinsed thoroughly under running tap water. The seeds were then left to soak in distilled water for 30 minutes. Thereafter, the seeds were sterilized by soaking in 70% (v/v) ethanol for 60 s, followed by double sterilization with 20% (v/v) commercial Clorox (containing 5% NaOCl) for 10 mins. The seeds were incubated for 14 days in petri dishes at 25 ± 2°C under the irradiance of cool-white fluorescent tubes with a 16 h photoperiod. The hypocotyl and leaf were then excised from the seedlings of 2 cm in height and the explants were used for callus induction. The surface sterilized seeds were used directly for callus induction. The basal medium used in the study was Murashige and Skoog (1962) with B5 vitamins (Gamborg et al., 1968). The basal medium was supplemented with 30 g/L of sucrose and 3

g/L of Gelrite®. The pH of the media was adjusted to a range of 5.75 to 5.80 using either 1 M of NaOH or HCl before autoclaved at 121°C and 15 psi for 20 minutes.

2.2 PGR optimisation of callus induction

The seed, leaf and hypocotyl explants were cultured on a 90 mm Petri dish containing full strength MS media supplemented with various combinations of PGRs at different concentrations. The effects two concentrations of BAP (0.5 and 1.0 mg/L) in combination with different concentrations of 2,4-D (0.5, 1.0 and 1.5 mg/L) were evaluated. Each treatment was prepared in three replicates containing five hypocotyl explants, ten leaf and seed plants respectively. The callus induction was conducted under the same condition as described above.

2.3 Elicitation

Compact callus with intense pink colour were selected for the elicitor treatment (tyrosine, copper sulphate and salicylic acid) to enhance betalain production. The elicitors were tested at four different concentrations respectively – 25, 50, 100 and 200 µM which were added to the MS media containing the optimized PGR concentration. The control plate was prepared in the absence of an elicitor in the media. Five clumps of 50 mg callus were cultured onto one Petri dish with three replicates for each treatment. All cultures were incubated for a period of four weeks under the same condition as described above.

2.4 Callus growth assessment

The callus growth assessments were carried out based on Castro et al. (2016) with some modifications. The growth of the callus cultures using different explants were assessed on a weekly basis for period of 4 weeks. The parameters taken include the percentage of callus induction, size, and colour. On the other hand, the growth assessment for callus treated with elicitors were carried out after four weeks of culture based on its colour, size, fresh weight, and callus type (friable or compact). The colour of the callus culture was determined visually and observed using a compound microscope (Olympus CX41, USA). Images were captured and analysed using the cellSens imaging software. The size of the callus in approximate area measurement (mm^2) were estimated using a graph paper. Callus from each treatment was blot dry on a tissue paper before fresh weight was measured. The dry weight of the callus treated with elicitors were performed by drying the callus in a hot air oven at 60°C until a constant weight was achieved (Suhu & Kalus, 2018).

2.5 Betalain extraction and quantification

The extraction and quantification of betalains from the callus cultures of *G. globosa* were carried out in accordance to Elbe (2001) and Bodhipadma et al. (2017). Callus was freshly harvested, ground, and extracted with 3 mL of distilled water on ice using pre-cooled mortar and pestle. The homogenate was made up to 5 mL and transferred to a centrifuge tube and centrifuged at 8000 rpm, 4°C for five mins. The collected supernatant was used to determine its absorbance value using a UV-VIS spectrophotometer (T60V, UK) at 476 nm, 538 nm, and 600 nm. The betalain contents were calculated based on the formula below:

Total betalains = Betacyanin content + Betaxanthin content

$$Betacyanincontent = \left(x \times \frac{DF}{1120}\right) \times n\frac{mg}{g}FWcallus$$

$$Betaxanthincontent = \left(x \times \frac{DF}{750}\right) \times n\frac{mg}{g}FWcallus$$

x = 1.095 × (a − c)

z = a −x

y = b − z − (x/3.1)
Abbreviations:

DF = dilution factor

FW = fresh weight

a = light absorption of the sample at 538 nm

b = light absorption of the sample at 476 nm

c = light absorption of the sample at 600 nm

n = weight of callus

x = light absorption of betacyanin

y = light absorption of betaxanthin

z = light absorption of impurities

2.6 Statistical analysis

The data obtained were analysed using one-way ANOVA (at p< 0.05) via SPSS version 26.0. The statistical differences among the means were compared using Duncan's New Multiple Range Test (DMRT) at 5% significance level.

Results and Discussion

3.1 Effect of different explants and PGRs on callus induction

3.1.1 Percentage of callus induction on different explants

Three types of explants which were the leaf, hypocotyl and seed were used for callus induction. Result showed that callus can be induced from different explants. They showed different responses towards different combinations of PGRs from week 1 to 4. (Table 3.1). On week 1, none of the explants showed callus formation. Starting from week 2, the leaf and hypocotyl of *G. globosa* started to show callus formation on all media supplemented with different PGRs combination whereby small swelling structures with red-pink colouration had appeared at the edges of the leaf and hypocotyl explants. Starting week 3, callus formation was observed on control media for leaf explants while callus begin to form from seed explants on PGR-containing media. There was no callus induced on the control media for both hypocotyl and seed explants at the end of week 4 (Table 3.1). In MS media supplemented with PGRs, callus was successfully induced from different explants were by the end of week 4. During the third and fourth week, light and dark pink calli were formed (Figure 3.1). Similar results were reported by Bodhipadma et al. (2017), there were no callus formation on PGR-free media from hypocotyl explants of all plant species tested (*Amaranthus cruentus* L., *Celosia argentea* var. *plumosa* (Burvenich) Voss and *G. globosa*).

The addition of PGRs resulted in a significantly higher percentage of callus induction from leaf (50.00 – 82.00%) and hypocotyl explants (68.00 – 92.00%) starting week 3, as compared to the control (0 – 44.00%). On week 4, there were no significant differences in the percentage of callus induction for the hypocotyl and leaf explants in all combinations of PGRs tested (Table 3.1). The finding was in agreement with Wahyuni et al. (2017) which reported that the addition of different PGRs (2,4-D, IBA and BAP) has no effect on the percentage of callus induction from *Justicia gendarussa* leaf as induction rate of 100% were recorded in all treatments. Research by Junairiah et al. (2018) also suggested that the alteration of concentrations of 2,4-D and BAP did not significantly affect the percentage of callus induction from the leaf of *Piper betle* L var Nigra. As for the seed explants, the media with 1.0 mg/L 2,4-D and 0.5 mg/L BAP produced the highest percentage of callus induction (53.33%) on week 4 which was significantly higher from other treatments (Table 3.1). In general, the percentage of callus induction from leaf and hypocotyl explants were much higher that seed explants indicating that both leaf and hypocotyl are suitable explants for callus induction as compared to seeds. In contrast, Mansur et al. (2018) has reported that addition of a high concentration of 2,4-D at 2.0 mg/L was able to induce high percentage of callus (85.00%) from chickpea seeds suggesting that higher concentration of PGRs could be needed to induce callus from seeds.

3.1.2 Effects of PGRs on the growth and production of pigmented callus

Analysis of callus growth was determined based on the size and colour. The size of the callus was recorded to determine which PGRs treatments were able to induce large callus as larger callus are often desirable for the mass production of secondary metabolites (Efferth, 2019). Results obtained showed that the *G. globosa* cultured in the PGRs containing media formed significantly larger callus as compared to the control media regardless of the types of explants used (Table 3.2). Nevertheless, there was no significant differences in the calli size among the PGRs tested in this study.

The formation of pigmented callus was observed and photographed in order to determine the suitable combination of PGRs to be used. Red to pink callus was expected to have higher concentration of betalain due to the presence of betacyanin, a red-violet subtype of betalain (Biswas et al., 2013). Among the tested explants, pink callus was only observed in callus derived from hypocotyl explants compared to leaf and seed explants (Figure 3.1 and Figure 3.2). Hence, calli derived from the hypocotyl explants were the most suitable to be used for betalain extraction and further sub-cultured for elicitation treatment. After 4 weeks of induction, the combination of 0.5 mg/L 2,4-D and 1.0 mg/L BAP resulted in a significantly higher percentage of pink callus which is 64.00% as compared to 8.00-36.00% in other PGR treatments in hypocotyl explants of *G. globosa* (Table 3.3). Similarly, the same concentration of 2,4-D and BAP was also found to produce the highest percentage of pigmented callus from hypocotyl explants of *Celosia* sp (Mastuti et al. 2021). In contrast to the findings of this study, the hypocotyl explants derived from 7-day-old *G. globosa* in-vitro seedlings did not result in pigmented callus formation in MS medium supplemented individually with 2,4D, BAP or NAA (Bodhipadma et al. 2017). In other words, exogenous supplementation of MS medium with auxin and cytokinin has been found to be a requirement for callus formation in some plant species including *G. globosa* as evidenced in this study (Ikeuchi et al., 2013).

3.2. Effect of elicitation on callus growth and betalain production

3.2.1 Callus size

The addition of copper sulphate resulted in larger callus size as compared to salicylic acid and tyrosine regardless of its concentration (Figure 3.3a). For instance, the size of the callus treated with 25 µM copper sulphate was about 13-folds (132 mm^2) larger than the first measurement of callus which is 9 mm^2 (Figure 3.4). However, as the concentration of copper sulphate increased to 200 µM, it did not improve the size further. A previous study showed that supplementation of copper at 2 µM effectively increased the callus growth of *Sorghum bicolor* but the growth decreased at high copper concentration (Nirwan & Kothari, 2003). Copper when used in excess cause phyotoxicity which then inhibits callus growth (Nirwan & Kothari, 2003; Taddei et al., 2007).

A slight increase in the callus size was recorded across different concentrations of tyrosine with no significance difference observed. Only salicylic acid applied at 50 µM showed significant difference in terms callus size when compared with 25 and 200 µM treatments. These results were different from the findings by Fadzliana et al. (2017). The addition of L-tyrosine was able to increase the size of the callus clumps compared to the callus grown with PGR alone. In another study, salicylic acid was reported to promote cell division. Babarabie et al., (2018) reported an increase in the stem cells of *Lilium longiflorum* when supplied with salicylic acid. Nonetheless, as higher concentration salicylic acid was provided, there was a cease in cell growth with a decrease in size. However, another study that examine the elicitation effect of salicylic acid on callus cultures of *Stevia rebaudiana* reported that bigger diameter and size of callus was obtained with higher salicylic concentration (Golkar et al., 2019). These results suggested that the effect of elicitation is highly species dependent.

Overall, 25 µM of copper sulphate recorded the largest callus and the smallest callus was produced under 200 µM of salicylic acid. The positive changes in the callus size as a result of elicitation with copper sulphate concurred with a few reports. For example, AL-Mayahi (2013) reported that the addition of copper sulphate into the MS media resulted in the highest proliferation rate of date palm callus as compared to the control callus. Similar findings were also reported by Yadav et al. (2011). A significant improvement was observed in the callus induction of *Hordeum vulgare L.* when copper sulphate was supplemented in the MS media. These indicated that copper sulphate was effective among the tested elicitors in increasing callus size of *G. globosa.*

3.2.2 Biomass of callus

Based on the results, it was observed that callus treated with different concentrations of salicylic acid and 100 µM copper sulphate had a lower fresh weight in comparison to the fresh weight of the control treatment (0.316 ± 0.111 g) (Figure 3.3b). On the other hand, the callus treated with 25 µM, 50 µM and 200 µM of copper sulphate had a significantly higher fresh weight than the control. Zarad et al. (2021) reported similar results where 1 to 2 mg/L of copper sulphate added to *Artemisia annua L.* callus cultures showed an increase in both fresh and dry weight. However, when the concentration of copper sulphate was increased, inhibition of the callus growth was observed. Dolgikh et al. (2017) further explained that copper can be toxic towards the plant cells when supplied at high concentrations.

The highest fresh weight was recorded in callus treated with 100 µM tyrosine with 0.703 ± 0.247 g followed by 25 µM copper sulphate with an average of 0.583 ± 0.237 g. Nevertheless, there is no significance difference between these two treatments. Sarab (2018) demonstrated that the addition of tyrosine to *Papaver somniferum* callus improved the fresh and dry weight significantly. However, contradicting results were reported by Winson et al. (2021) whereby the addition of tyrosine significantly reduces the fresh and dry mass of *Hylocereus costaricensis* callus. The tolerance level of tyrosine concentration on the proliferation of callus culture is species specific and over the course of years varying results has been observed in different species of plants (Kleinowski et al., 2014). On the other hand, the lowest fresh weight was recorded by callus treated with 100 µM salicylic acid with 0.183 ± 0.018 g and it was significantly lower than the fresh weight of callus from the control 0.316 ± 0.111 g.

Results showed that the dry weight recorded showed a similar pattern to the fresh weight where the highest dry weight was in callus treated with 100 µM tyrosine, 0.069 ± 0.029 g and the lowest was observed in treatment with 100 µM salicylic acid with 0.020 ± 0.002 g (Figure 3.3c). The results suggested that salicylic acid had negative effects on the callus fresh and dry weight. A study by Bulgakov et al. (2003) showed that growth of callus cultures of *R. cordifolia L*. (Rubiaceae) was inhibited, when treated with 100 µM salicylic acid. It was further explained that the salicylic acid may have imposed stress on callus growth development resulting in a low accumulation of biomass.

3.2.3 Morphological appearance and betalain profile of callus treated with different types of elicitors.

The betalain content of the callus culture treated with different elicitors were measured and compared to the control. From the morphological appearance, we could clearly see that callus treated with tyrosine had the most intense pink colour as compared to other elicitors (Figure 3.5, 3.6 and 3.7). The pink pigments were distributed throughout the callus clumps across the different concentrations of tyrosine. On the contrary, the callus from the control treatment showed yellow and a slight green colouration. Salicylic acid produced light pink pigments mostly on the edges of the friable callus (Figure 3.6d-j). Similarly, only light pink was observed in callus treated with copper sulphate and the callus generally appeared as yellow.

Results from the betalain quantification showed that both betacyanin and betaxanthin were detected in the callus from all treatments (Figure 3.8). Generally, the tyrosine treatment resulted in higher total betalain content as compared to the other two elicitors. The highest betalain content was found in callus cultured on MS medium supplemented with 100 µM of tyrosine. These results coincide with the findings by Fadzliana et al. (2017) where the addition of 20 mg/L of L-tyrosine showed 1.5-fold higher production of betacyanin in red pitaya callus. Wang et al. (2017) also reported that as the concentration of tyrosine increases, higher production of betacyanin were observed in red beets. These findings could be explained as tyrosine is the precursor for stimulating the biosynthesis pathway for the conversion of the respective betalain compounds and in this case, it would be the betacyanin content (Gandía-Herrero & García-Carmona, 2013).

In this study, a higher total betalain content was observed in callus treated with low concentration of salicylic acid (25 µM) supplied. Further increase in the salicylic acid concentration had a negative effect on the amount of betalain produced. Also, the callus treated with salicylic acid produced a higher betaxanthin content and a lower betacyanin content. Friable pink callus was observed for red pitaya with supplementation of salicylic acid where higher betacyanin percentage were obtained with lower concentration of salicylic acid (Wee et al., 2018). Similar results were observed in *Alternanthera tenella* where the supplementation of low concentration of salicylic acid significantly increased the overall betacyanin content (Kleinowski et al., 2014).

Lastly, almost equal ratio of betacyanin and betaxanthin was observed from 25 µM copper sulphate treatment. This could be due to the light pink to orange hues observed on the callus when treated with copper sulphate (Figure 3.7d-j). These findings coincide with the study conducted by Warhade & Badere, (2018) where the betaxanthin content in the callus of *Celosia cristata* was doubled as a result from the copper sulphate elicitation suggesting that copper sulphate could enhance the betaxanthin production by either enhancing the activity of the enzyme involved in the synthesis of betaxanthin or by hindering the activity of enzymes involved in betacyanin synthesis.

Conclusion

In conclusion, callus was successfully induced from the leaf, hypocotyl and seed explants of *G. globosa*. Among them, the percentage callus induction was high in leaf and hypocotyl explants (84.00% to

100.00%) in MS medium supplemented with BAP and 2,4-D. The different types of explants showed different responses towards the supplementation of different levels of PGRs and elicitors. *G. globosa* formed larger callus in all PGRs treatments given (2.47 mm^2 to 18.21 mm^2). Higher percentage of pink callus was obtained from the hypocotyl of the plant while the leaf and seed explants did not produce any pink callus. Results from the elicitation with different elicitors showed that 100 µM tyrosine was effective in enhancing the betacyanin content of the callus followed by salicylic acid. In contrast, elicitation with copper sulphate produced callus with bigger size in comparison to the other elicitors. Nevertheless, copper sulphate did not enhance the total betalain content. With the optimised in-vitro callus induction and betalain production, this study can be further extended to establish cell suspension cultures for commercial production. In this sense, *G. globosa* is a promising source of betalain production.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; DW, Dry weight; FW, Fresh weight; PGRs, Plant growth regulators;

Declarations

Conflict of interest

The authors declare no conflict of interest.

Data availability statement

The authors declare there is no associated data.

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Tables

Table 3.1

Callus induction period	Treatment / Type of explant	Percentage of induction (%) Control	0.5 mg/L 2,4-D + 0.5 mg/L BAP	1.0 mg/L 2,4-D + 0.5 mg/L BAP	1.5 mg/L 2,4-D + 0.5 mg/L BAP	0.5 mg/L 2,4-D + 1.0 mg/L BAP	1.0 mg/L 2,4-D + 1.0 mg/L BAP	1.5 mg/L 2,4-D + 1.0 mg/L BAP
Week 1	Leaf	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Hypocotyl	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Seed	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 2	Leaf	0.00 ± 0.00a	10.00 ± 17.32a	6.00 ± 8.94a	6.00 ± 8.94a	2.00 ± 4.47a	2.00 ± 4.47a	2.00 ± 4.47a
	Hypocotyl	0.00 ± 0.00a°	24.00 ± 32.86a°	24.00 ± 21.91a°	4.00 ± 8.94a°	24.00 ± 21.91a°	20.00 ± 14.14a°	24.00 ± 16.73a°
	Seed	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Week 3	Leaf	44.00 ± 40.37b	82.00 ± 14.83a	50.00 ± 33.91ab	72.00 ± 16.43ab	74.00 ± 19.49ab	70.00 ± 7.07ab	78.00 ± 20.49ab
	Hypocotyl	0.00 ± 0.00b°	88.00 ± 17.89a°	88.00 ± 17.89a°	76.00 ± 21.91a°	92.00 ± 10.95a°	72.00 ± 30.33a°	68.00 ± 30.33a°
	Seed	0.00 ± 0.00b*	30.00 ± 10.00a*	36.67 ± 20.82a*	36.67 ± 5.77a*	16.67 ± 15.28ab*	33.33 ± 20.82a*	30.00 ± 10.00a*
Week 4	Leaf	52.00 ± 48.68b	94.00 ± 8.94a	84.00 ± 21.91ab	98.00 ± 4.47a	90.00 ± 17.32a	92.00 ± 13.04a	96.00 ± 8.94a
	Hypocotyl	0.00 ± 0.00b°	90.00 ± 14.14a°	94.00 ± 13.42a°	98.00 ± 4.47a°	92.00 ± 10.95a°	94.00 ± 8.94a°	94.00 ± 13.42a°
	Seed	0.00 ± 0.00b*	43.33 ± 15.28ab*	53.33 ± 11.55a*	46.67 ± 5.77ab*	30.00 ± 10.00b*	40.00 ± 20.00ab*	50.00 ± 10.00ab*

Values represent mean SE of five replication (leaf and hypocotyl) and three replication (seed) and different letters indicate their relative significance at $p<0.05$ probability level.

Table 3.2

Treatment / Type of explant	Size (mm^2) Control	0.5 mg/L 2,4-D + 0.5 mg/L BAP	1.0 mg/L 2,4-D + 0.5 mg/L BAP	1.5 mg/L 2,4-D + 0.5 mg/L BAP	0.5 mg/L 2,4-D + 1.0 mg/L BAP	1.0 mg/L 2,4-D + 1.0 mg/L BAP	1.5 mg/L 2,4-D + 1.0 mg/L BAP
Leaf	1.39 ± 1.40b	18.21 ± 6.12a	16.22 ± 5.14a	15.66 ± 3.14a	15.68 ± 3.45a	14.43 ± 4.77a	14.78 ± 5.11a
Hypocotyl	0.00 ± 0.00b°	9.56 ± 4.13a°	8.84 ± 3.53a°	9.92 ± 4.16a°	13.16 ± 5.85a°	9.64 ± 4.99a°	9.14 ± 5.09a°
Seed	0.00 ± 0.00b*	2.47 ± 0.81a*	2.57 ± 0.50a*	1.57 ± 0.75ab*	1.97 ± 0.55a*	2.00 ± 1.51a*	2.13 ± 0.84a*

Values represent mean SE of five replication (leaf and hypocotyl) and three replication (seed) and different letters indicate their relative significance at $p<0.05$ probability level.

Table 3.3

Treatment	Percentage of pink callus (%) Control	0.5 mg/L 2,4-D + 0.5 mg/L BAP	1.0 mg/L 2,4-D + 0.5 mg/L BAP	1.5 mg/L 2,4-D + 0.5 mg/L BAP	0.5 mg/L 2,4-D + 1.0 mg/L BAP	1.0 mg/L 2,4-D + 1.0 mg/L BAP	1.5 mg/L 2,4-D + 1.0 mg/L BAP
Hypocotyl	0.00 ± 0.00c°	8.00 ± 17.89bc°	24.00 ± 35.85bc°	16.00 ± 26.08bc°	64.00 ± 16.73a°	36.00 ± 26.08ab°	36.00 ± 26.08ab°

Values represent mean SE of five replication (leaf and hypocotyl) and three replication (seed) and different letters indicate their relative significance at $p<0.05$ probability level

Figures

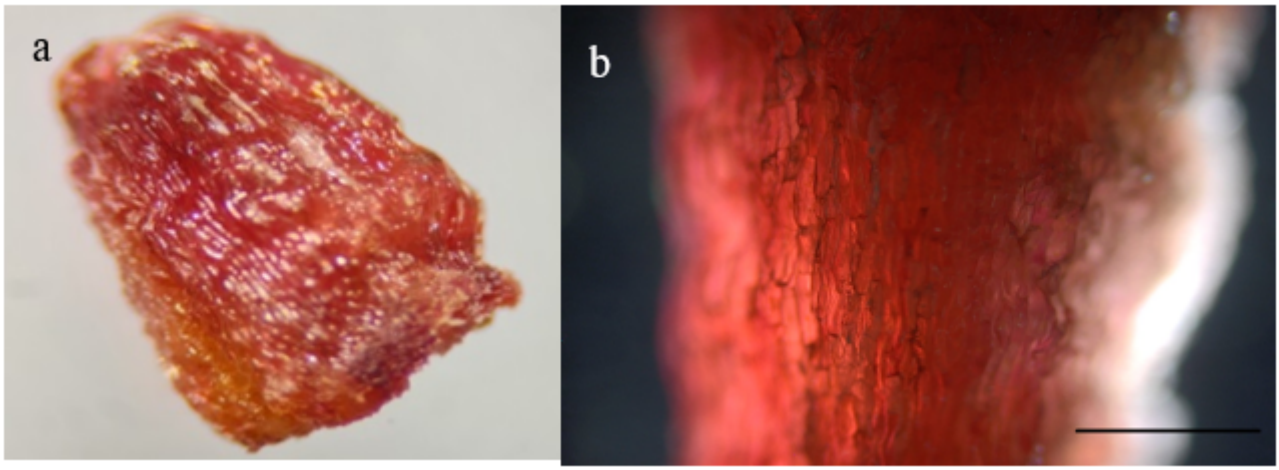

Figure 1

Figure 3.1. Callus induced from *G. globosa* hypocotyl using different PGR treatments. (a-b: pink callus). Bar represents 1 mm.

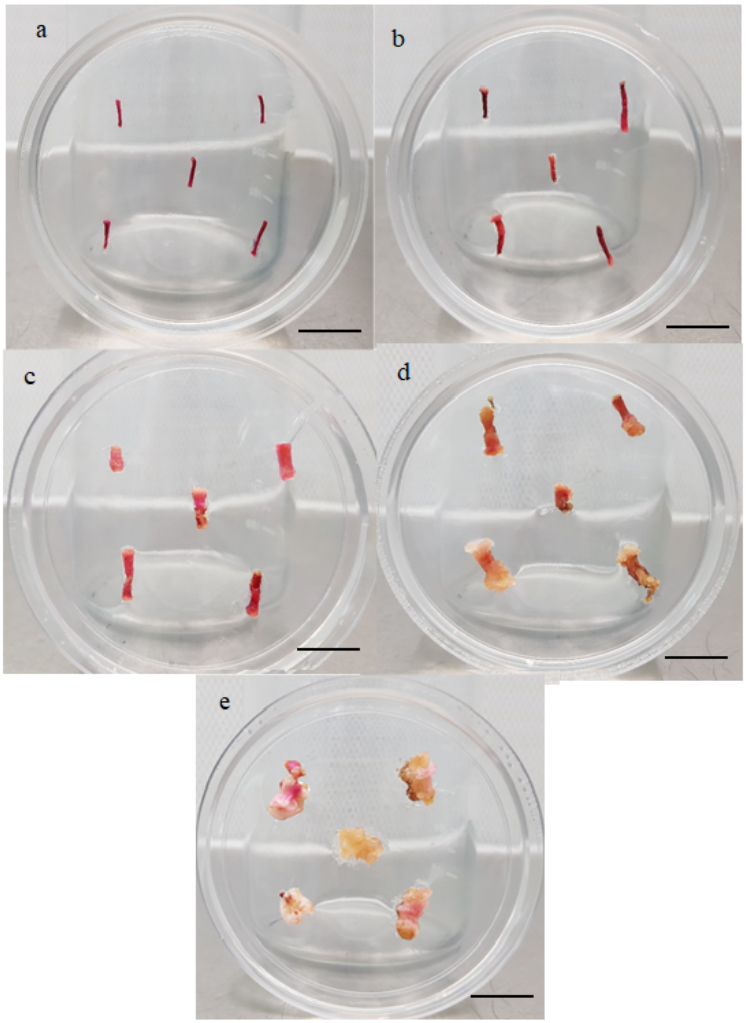

Figure 2

Figure 3.2. Callus induction from the hypocotyl of *G. globosa*.(a: first day; b: first week: c: second week; d: third week: e: fourth week). Bar represents 1 cm.

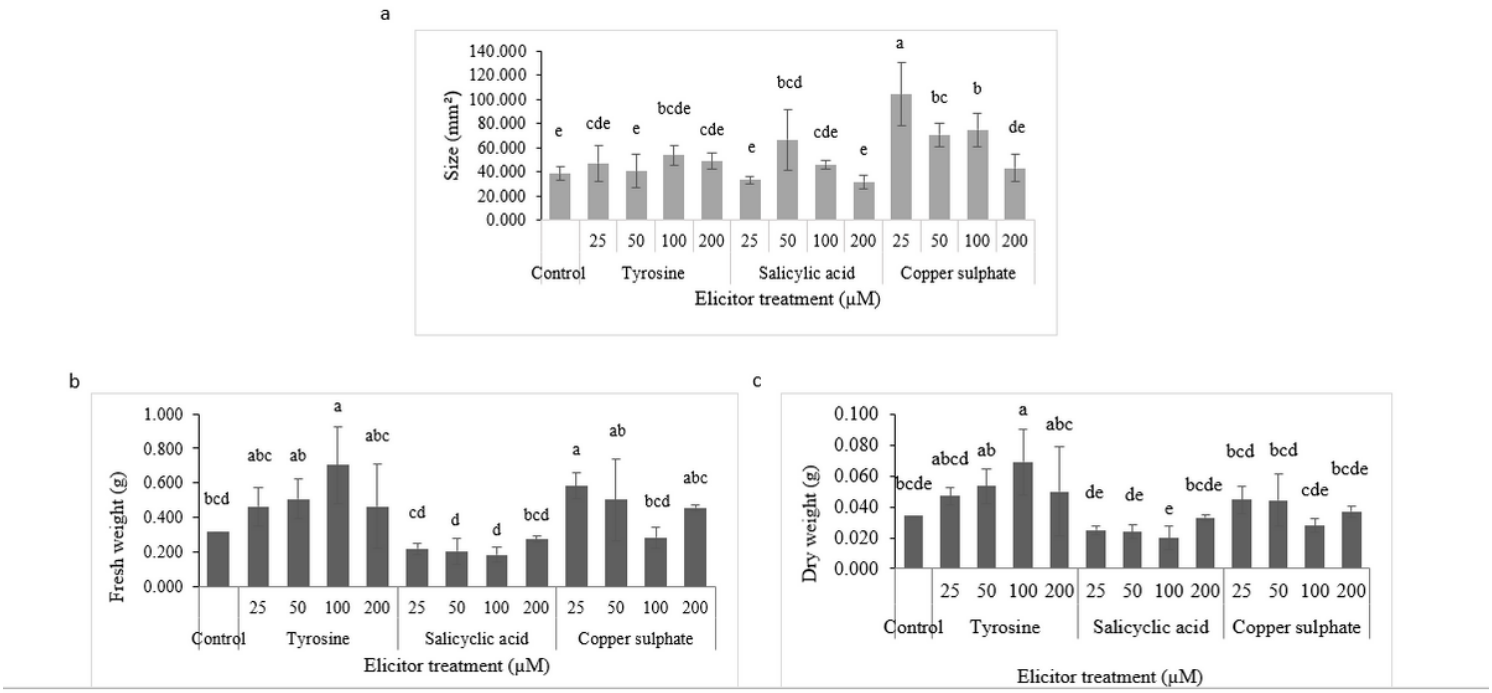

Figure 3

Figure 3.3: Growth changes of callus after four weeks of treatment with different elicitors. (a): Size, (b): Fresh weight, (c): Dry weight

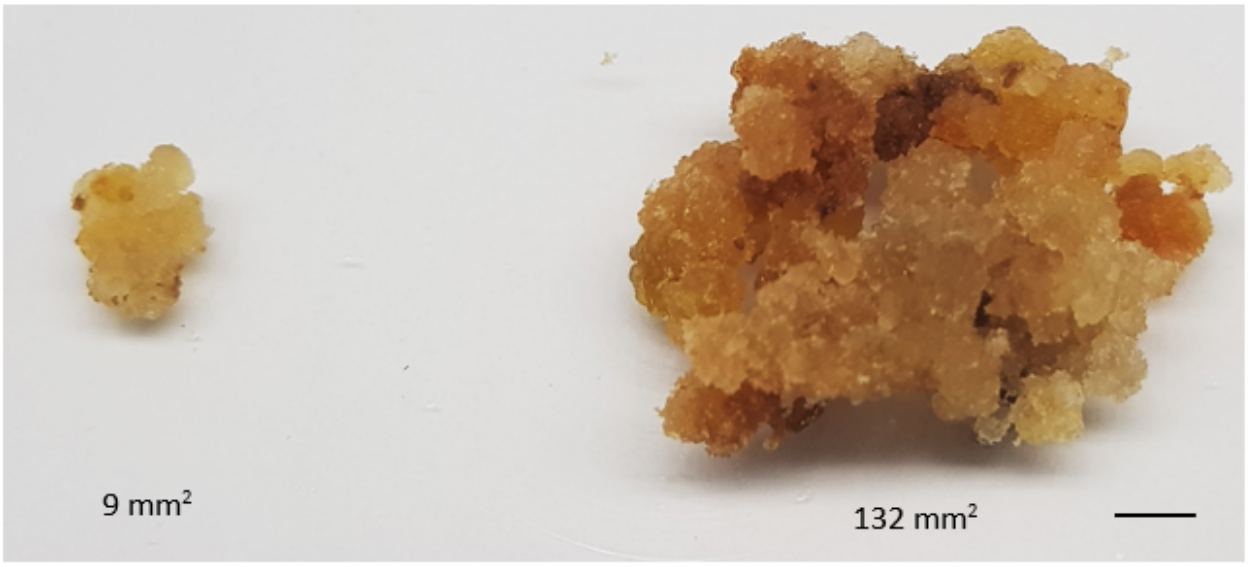

Figure 4

Figure 3.4: Changes in callus size as a result of $CuSO_4$ elicitation at 25 µM. Scale bar represents 1 cm.

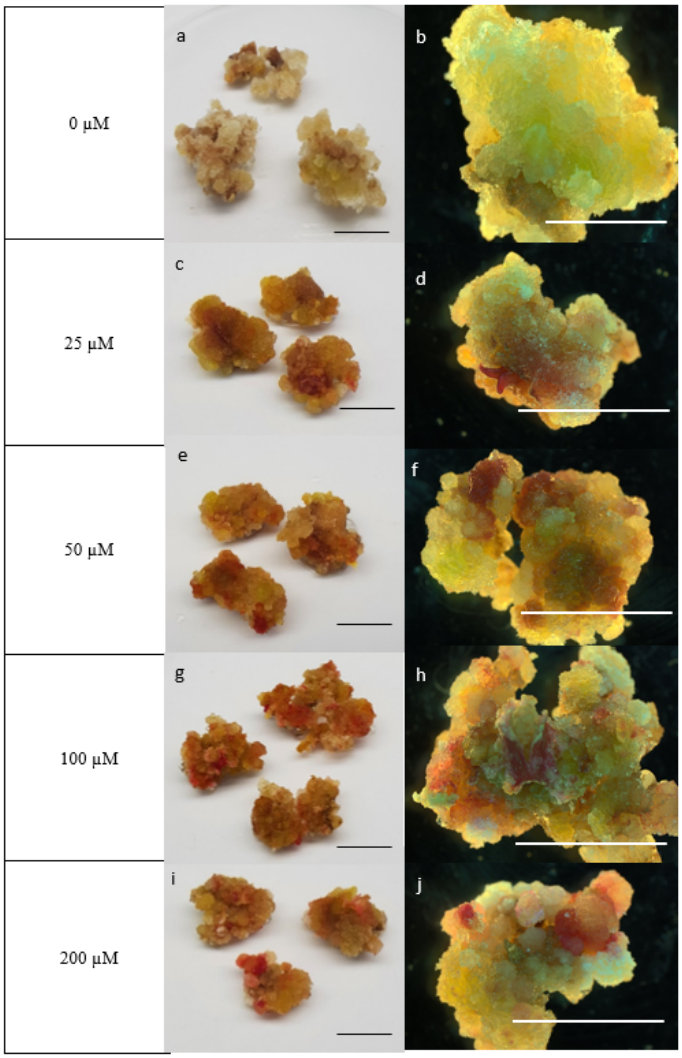

Figure 5

Figure 3.5: Morphological appearance of callus culture after four weeks of elicitation with various concentrations of tyrosine. Scale bar represents 1 cm.

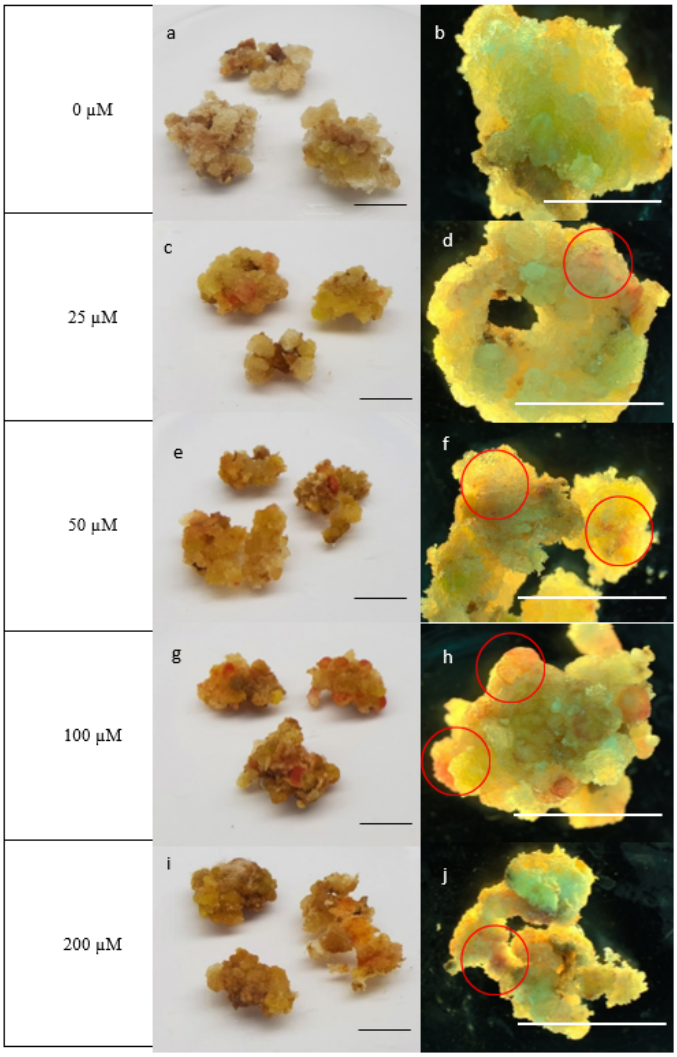

Figure 6

Figure 3.6: Morphological appearance of callus culture after four weeks of elicitation with various concentrations of salicylic acid. Scale bar represents 1 cm. Red circles indicate formation of pink callus.

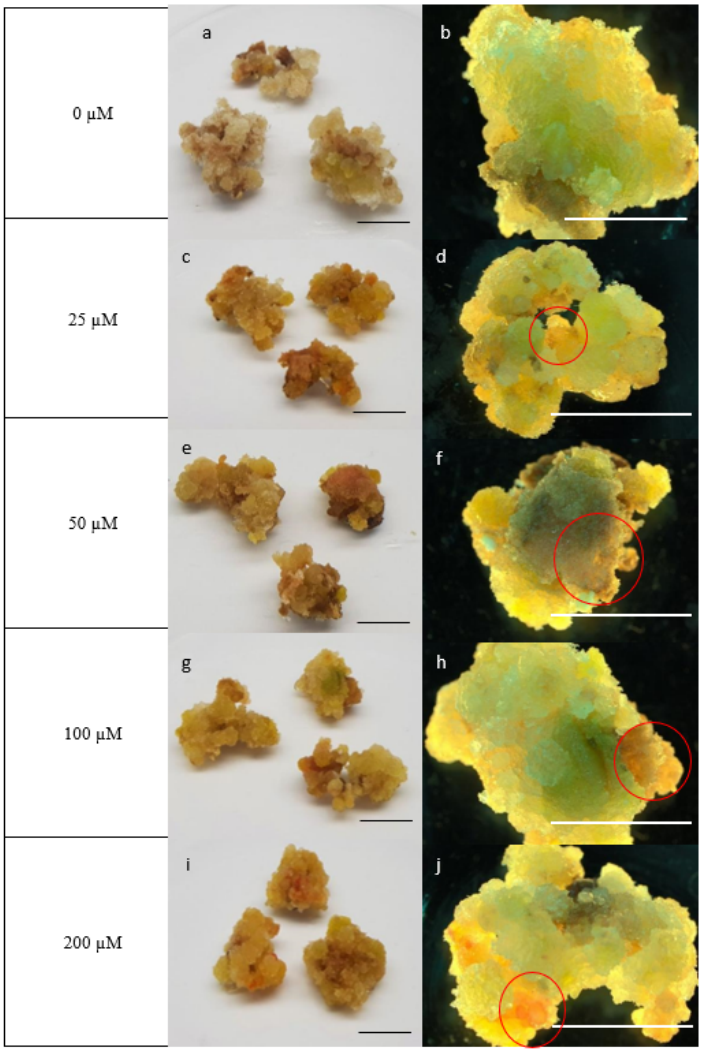

Figure 7

Figure 3.7: Morphological appearance of callus culture after four weeks of elicitation with various concentrations of copper sulphate. Scale bar represents 1 cm. Red circles indicate formation of pink callus.

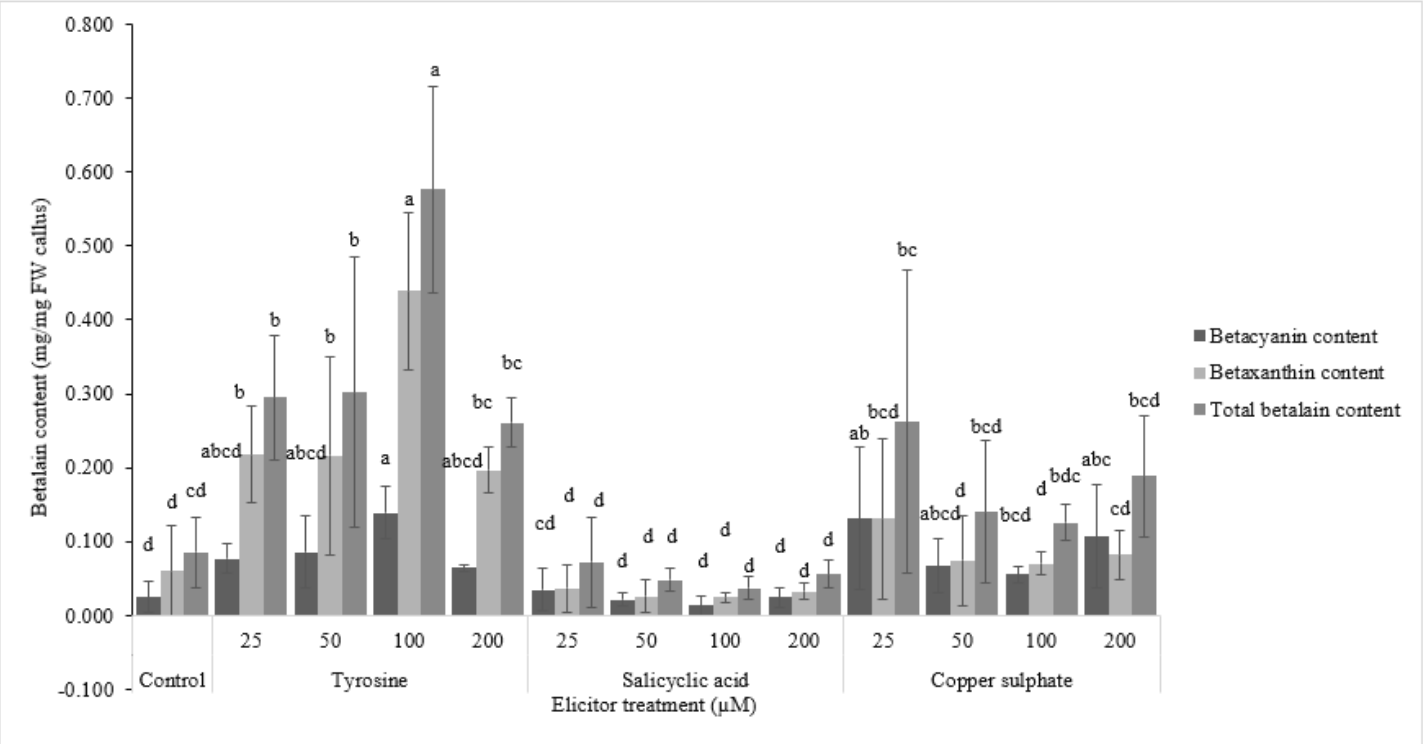

Figure 8

Figure 3.8: Betalain content of G. globosa callus treated with different elicitors. The bars are represented by the mean-SE of three replicates where the letters indicate their relative significance level at 5%.