

UV-Rays induced Cytomorphological and biochemical variations in *Stevia rebaudiana* Bertoni

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Abstract

Stevia rebaudiana Bertonii is a perennial herb of the *Asteraceae* family and is highly medicinal in number of ways. Abiotic stress such as UV radiation exposure was reported to have a beneficial effect on morphological traits of the plant. Plantlets of *Stevia* (4–5 cm) were exposed to UV-B rays with differential time viz. 20, 40, 60, 80, and 100 minutes, respectively along with the control set. The exposed plantlets were transferred to the greenhouse for further study and growth. After 45 days of plantation, morphological studies were taken out and tabulated. At intermediate doses *i.e.*, 40 and 60 min, an increase in leaf area, leaf width, leaf length, plant height, internodal length and width of stem have been found in relation to morphological traits while in case of cytological study different chromosomal arrangements were observed *viz.* scattering, precocious movement, stickiness, unorientation and bridge formation, *etc.* Hence on the basis of morphological and cytological studies performed on *Stevia* after UV irradiation, it was concluded that, shorter exposure of UV-B (40 and 60 min) induced some better morphological traits so that the qualitative and quantitative features of the plant were enhanced.

Introduction

Stevia rebaudiana also known as Sweet-leaf and Candyleaf plant is a very famous plant of family *Asteraceae* (chromosome number $2n = 22$) due to its high medicinal value among all other plants of the same family. It is very high in sugar contents and numerous other metabolites which are very much considerable for the medicinal industry and welfare of humans (Yadav *et al.*, 2011). The plant has numerous medicinal properties including antidiabetic, anticancer, antimicrobial, antiobesity, antihypersensitivity, antihyperglycemia, tooth and heart caring properties. Plant is currently trending among the scientific community as it has a very important chemical property of having sweet glycosides which are very much sweet in nature than table sugar (100–300 times), these glycosides mainly consist of Steviosides and Rebaudiosides found concentrated naturally in the leaves of the plant (Jeppensen *et al.* 2002, 2003; Chan *et al.* 1998). Stevioside (St) is the main sweet compound that can be extracted from the *Stevia* plant to be used as sweeteners to sugars in several countries as calorie-free sweeteners such as Japan, China, and Brazil of benefit to diabetics and those wishing to reduce sugar intake health reasons (Midmore and Rank, 2002). *Stevia* becomes more prevalent in several developing and developed countries as a significant crop for the nontoxic, non-nutritive, high-potency production of sweeteners. The natural sweet properties of *Stevia* are characterized by the chemical composition of diterpene glycoside which includes primarily stevioside, stevioside, glucoside, and rebaudioside A to E (Gupta *et al.*, 2013). Worldwide demand for both purified glycosides and dried leaves of *Stevia* is gradually increasing (Angelini *et al.*, 2016).

Stevia is an introduced plant for India as it is a native plant of Paraguay (South America). The plant has a number of medicinal activities which were reported in numerous literatures but the major advantage of the plant is its natural glycosides (Stevioside and Rebaudiosides) which are 100–300 times sweeter than normal table sugar and interestingly do not get metabolised in our body to affect our cellular blood glucose level (DuBois, 2000; Goyal *et al.*, 2010). As it is an introduced plant, hence major concern arisen with the time in case of this plant is its continuously reducing genetic variability and vigour simultaneously due to continuous selfing of the same. Here in the study, we have tried to get rid of the problem with the help of induced mutagenesis technique which is very famous of inducing numerous genetic variabilities and more favourable vigour in the plants (Goyal *et al.*, 2010; Yadav *et al.*, 2011).

Mutation breeding is a method that changes plant gene pools to accelerate the enhancement of plants considerably with anticipated traits in comparison with conventional approaches for breeding, numerous mutagenic agents *e.g.* physical ones such as X-rays, gamma-rays, thermal neutrons, fast neutrons, or chemicals like sodium azide (SA), ethyl methane sulfonate (EMS), diethyl sulfate (DES), methyl nitrosourea (MNU) could be utilized to give beneficial mutations (Toruan-Mathius *et al.* 1995; Yadav *et al.*, 2011).

Here in the study, we have applied UV-B to plant to enhance the genetic variability and morphological and biochemical vigour too because number of studies have shown significant UV-B radiation effects on barley growth parameters including stem height, sprout count, leaf area, and biomass (Correia *et al.*, 1999; Nasser, 2001). Hakala *et al.* (2002) determined the sensitivity of various agricultural plant species including barley, wheat, oat, clover, timothy, fescue, and potato to UV-B radiation exposure (as if the ozone layer would be decreased by 30%) and found no significant variation of biomass accumulation or yield. Ziska and Teramura (1992) and Dai *et al.* (1994) reported that prolonged exposure to UV-B light affects plant height, leaf area, dry weight, net assimilation rate, and relative growth rate in some rice cultivars.

Abiotic stressors such as UV radiation exposure was reported to have a beneficial effect on the production of secondary metabolites. Phenolic compounds are secondary metabolites that have been widely distributed in plants and are important constituents of the human diet and also serve as defence mechanisms against biotic and abiotic stresses (Bhattacharya *et al.* 2010). Phenolic compounds as antioxidants have the ability to destroy free radicals due to their hydroxyl group (Pourreza, 2013).

Several studies revealed that exposure to lower or intermittent/pulsed levels of UV-B radiation plays hermetic effects (Höllet *et al.*, 2019; Kumari *et al.* 2009), while excessive, continuous UV-B exposure induces harmful and damaging effects on the plants (Rai and Agrawal, 2020). To understand these cost-benefit relationships among the abiotic stressors, *i.e.*, UV-B rays and plants, several studies have been done focusing mainly on the single acute short-term or long-chronic UV-B exposures (Kumari *et al.*, 2009; Tripathi *et al.* 2011).

Currently, stratospheric ozone layer is continuously depleting because of increasing atmospheric pollutant sources such as bromine- and chlorine-containing compounds has led to an increase in ultraviolet-B radiation (UV-B) reaching the earth's surface (Bjorn *et al.*, 1997; McKenzie *et al.*, 2003). Stratospheric ozone recovery may possibly be delayed due to a number of uncertainties, including interaction with other projected changes in global climate such as global warming (3,4). Therefore, it still remains interesting to investigate the effects of elevated UV-B radiation on various aspects of plant growth. (Wang *et al.*, 2010)

The study was hypothesized to find out which UV-B exposure is helpful to plant defence strategies and accumulation in different plant parts and to broaden the narrow genetic base of the plant too. The study also aims to justify the effect of increasing concentration of UV-B radiation in the environment. To fulfil the hypothesis, present study estimates the response of *Stevia* plant against different time duration exposure of UV-B rays.

Materials and method

Experimental design and procurement of materials

Plantlet of *Stevia rebaudiana* Bertonii collected from the Central Institute of Medicinal and Aromatic Plants (CIMAP), Lucknow, Uttar Pradesh. Plantlets were sown in a triplicate manner five different sets for different exposure times viz. Set A for 20 min, Set B for 40 min, Set C for 60 min, Set D for 80 min, Set E for 100 min, and set F as control.

UV-B treatment

Sets A, B, C, D and E were kept in an Ultraviolet fluorescence chamber and the seedlings were irradiated with a UV-B lamp (CX-20 equipped with internal 8-watt tubes) for 20, 40, 60, 80, and 100 min respectively and F was assigned as control set. After completion of irradiation, they were kept for recovery for half an hour, and after UV-B exposure plantlets were transplanted to green house chamber.

Morphological analysis

Plant height and Internodal length

Plant height was taken after 45 days with a measuring scale and internodal length was measured when the plant height attained stability.

Stem Width

Stem width is measured with the help of vernier callipers.

Leaf Length and Leaf Width

The Leaf length and width were also measured with the help of a normal 30 cm scale.

Leaf area

leaf area of randomly selected leaves along with control was measured by using "Systronics leaf Area Metre 211" which is easy and accurate.

Cytological analysis

Meiotic study

Young floral buds of the plants were taken and fixed simultaneously when they started coming out. For studying cytology of the plant, young floral capitula of *Stevia* were fixed in Carnoy's fixative for 24 hr and then transferred in alcohol for preservation. For the meiotic study anthers were excised from floral capitula and kept on a slide. Anther was teased and stained in 2% acetocarmine stain followed by squash preparation. Meiotic slides were observed under the Nikon Eclipse E200 microscope and pollen fertility was evaluated by an acetocarmine stainability test.

Pollen fertility

Pollen fertility was observed and calculated on the basis of staining with acetocarmine stain. The fertile one takes stain while sterile one remains unstained.

Biochemical analysis

Biochemical test was also done for getting amount of *Chl a*, *chl b*, carotenoids and for estimation of different glycosides responsible for sweetness of the leaves of the plant. The estimation of *chl a*, *chl b* and carotenoids was made using "Systronics UV-Vis Spectrophotometer".

Statistical analysis

All the data were taken in three replicates for the accuracy of results. By using SPSS 16.0 software analysed the dataset. ANOVA was used as statistical tool for mean variation. The graphical figure of data was plotted in Sigma Plot 10.0 Software.

Formula used

$$1. \text{ Total abnormality percentage (TAB \%): } \frac{\text{Total number of abnormal cells}}{\text{Total number of cells observed}} \times 100$$

$$2. \text{ Pollen fertility: } \frac{\text{Number of stained pollen}}{\text{total number of pollen}} \times 100$$

$$3. \text{ Chlorophyll a: } \frac{12.25(A_{665}) - 2.79(A_{665}) \times \text{volume}}{\text{weight of leaf tissue (mg)}}$$

$$4. \text{ Chlorophyll b: } \frac{21.5(A_{665}) - 5.1(A_{665}) \times \text{volume}}{\text{weight of leaf tissue (mg)}}$$

5. Carotenoids:
$$\frac{[1000(A) - 1.82(Chla) - 85.02(Chlb)] / 198 \times volume(ml)}{weight of leaf tissue(mg)}$$

Results and Discussion

In the present study, we have found that exposure to UV-B rays on *Stevia* plantlets leads to different changes in cytological, morphological and biochemical parameters. It was found that stem (Table 1 and Fig. 1A) and leaf (Table 1 and Fig. 1B) of *Stevia* have a different response at the morphological level and at cytological level it produces a large number of anomalies at the metaphase and anaphase stages of meiotic cells and sterility in pollen cells (Table 2 and Fig. 2). In biochemical study the photosynthetic pigments (*Chla*, *Chlb*) as well accessory pigments (Carotenoids) have shown a different pattern of difference in their concentrations (Table 3 and Fig. 3) depending upon the exposure period of UV-B on the plants.

Morphological:

In case of morphology of the plants the treated sets have shown a dramatic outcome. In the results we have observed that at intermediate doses (*i.e.*, 40 min and 60 min) plants have shown positive responses in terms of vegetative growth in stem and leaves. The plant height noted highest (79.43±2.43) in 40 min treated set while lowest (36.30±1.53) was observed at 100 min. Similarly in case of internodal length highest (10.60±0.21) was observed at 40 min and lowest (7.47±0.26) was at 100 min. similar results were obtained in case of leaf area also. Highest leaf area (27.16±0.50) noted in 40 min treatment while lowest one (11.30±0.25) was observed at 100 min.

These above-mentioned positive changes or enhanced vigor of the plants at intermediate doses or lower doses (40 min and 60 min) may be found due to priming effects of UV-B rays. These priming effects lead to activation of several factors related to stress tolerance and increased production of phytohormones which are responsible for increase in plant height and other positive morphological responses such as increased stem width, leaf length, width and leaf area *etc.* (Kumar and Mishra, 2019; Thomas and Puthur 2020). Delibaltova and Ivanova (2006) have also found in their study that low exposure of UV irradiation is a safer methodology to enhance the quality and productivity of the plants. According to Kacharava *et al.* (2009) increase in plant height and internodal lengths take place due to regulation in photomorphogenic genes such as, HY5 and COP1 *etc.*, which play a crucial role in the growth and development of plants. In some studies, it was also found that the UV-B exposure doesn't disturb the morphology of the plants but in maximum cases it causes some differences (Hidema *et al.*, 2007; Du *et al.*, 2011).

No./ Character	Stem length (cm)	Stem width (cm)	Internodal length (cm)	Leaf length (cm)	Leaf width (cm)	Leaf area (cm ²)
Control	51.33±0.44	0.90±0.06	8.57±0.20	5.20±0.17	2.97±0.15	17.16±0.37
20 Min.	53.63±0.98	0.93±0.09	9.43±0.12	5.67±0.24	3.20±0.17	20.46±0.52
40 Min.	79.43±2.43	1.20±0.12	10.60±0.21	6.57±0.34	3.53±0.09	27.16±0.50
60 Min.	75.00±1.12	1.57±0.15	9.97±0.09	5.70±0.29	3.33±0.24	23.13±0.20
80 Min.	50.57±1.60	0.63±0.12	8.43±0.15	5.43±0.19	2.77±0.09	14.25±0.53
100 Min.	36.30±1.53	0.47±0.09	7.47±0.26	4.70±0.21	2.03±0.09	11.30±0.25

Table 1: Representing the data of morphological parameters

Cytological:

It is well known that UV-B has enough potential to disturb the chromosomal structure and function by disturbing the DNA material of the organism (Gill *et al.*, 2015). In present study we have found normal meiosis (2n=22) in non-treated plants while we have investigated and observed a number of abnormalities in treated sets *viz.* scattering, stickiness, precocious movement unorientation, laggard, disturbed polarity, *etc.* (Figure 3). In the study, it was recorded that as the exposure time increases, the total abnormality percentage (TAB%) is also increasing simultaneously due to the chronic effect of UV-B on the cellular functioning of the plants. The lowest TAB% (8.12±1.00%) was found on lowest exposure *i.e.*, 20 minute, while highest (13.35±0.58%) was noted at highest exposure *i.e.*, 100 minute (Table 2, Fig 2). The most common abnormality which was found in the meiotic study was scattering and stickiness at metaphase and anaphase stage of meiosis. Similar cytological results have also been found in number of studies related to UV-B treatment, including Kumar and Pandey (2017) in Coriander, Kumar and Mishra (2019) in Bhingraj and Kumar and Bhardwaj (2019) in Cumin.

The anomalies observed in the study may be attributed to several factors such as **stickiness** generally arises due to improper folding of the chromosomal fibre into a single chromatid and the chromosomes become attached to each other by a sub-chromatid bridge (McGill *et al.* 1974; Klasterka *et al.*, 1976). **Chromosomal bridges** are arising may be due to the chromosomal breaks and their joining (Dhulgande, 2015; Liu *et al.*, 2015). A **laggard** in chromosomes may appear due to delayed terminalisation, chromosome stickiness, or failure of chromosome movement or due to differential speed of movement of chromosomes (Qian, 2004; Muniswamy Reddy and Munirajappa, 2012).

Another abnormality observed was the **precocious movement** of chromosomes, which may be due to the migration of chromosomes to the poles which can result in early chiasma terminalization in diakinesis or metaphase I. (Srivastava and Kapoor, 2008). **Scattering** and **unorientation/disturbed polarity** of chromosomes are appearing there due to disturbance or loss of spindle fibres (Kumar and Rai, 2007; Kumar and Bhardwaj, 2019).

The UV-B treatment also led to decrement in viable pollen grains. A number of studies suggested that *Stevia* already have a low viability in terms of pollen grains *i.e.* 65% around (Montiero, 1980) and the viability decreases more and more with increasing exposure time. Highest pollen fertility (65.87%) was found in control plants while lowest (37.70%) was observed at highest exposure time. Chromosomal aberrations are the probable reason for pollen sterility (Bhat *et al.*, 2007). Generation of reactive oxygen species and hydrogen peroxide may also be behind this outcome of pollen sterility (Heet *et al.*, 2006).

Table 2: Representing percentage of various cytological anomalies including Metaphasic and Anaphasic abnormalities and the total abnormality percentage of the UV-treated sets in comparison to control set

No. of PMCs Observed	Metaphasic Abnormality (Mean±SE)				Anaphasic Abnormality (Mean±SE)						Oth	TAB%
	Sc	Pm	St	Un	Lg	Un	Bg	St	Dp	Pr		
65	-	-	-	-	-	-	-	-	-	-	-	-
63	0.64±0.26 ^a	0.51±0.12 ^a	1.91±0.40 ^a	0.51±0.26 ^a	0.38±0.22 ^a	1.52±0.21 ^a	0.76±0.23 ^a	0.76±0.01 ^a	0.38±0.22 ^a	0.50±0.33 ^a	0.25±0.13 ^a	8.12±1.00
53	1.19±0.25 ^{ab}	0.53±0.13 ^{ab}	1.85±0.37 ^a	0.79±0.40 ^a	0.26±0.26 ^a	1.72±0.38 ^{ab}	0.66±0.13 ^b	0.78±0.39 ^a	0.65±0.12 ^a	0.54±0.36 ^a	0.53±0.13 ^a	9.51±0.81
48	1.48±0.35 ^{ab}	1.04±0.46 ^{ab}	2.62±0.31 ^a	0.66±0.36 ^a	0.67±0.35 ^a	2.63±0.32 ^{ab}	0.39±0.22 ^b	0.53±0.26 ^a	0.65±0.47 ^a	0.41±0.41 ^a	0.52±0.13 ^a	11.61±0.7
39	1.24±0.23 ^{ab}	1.64±0.21 ^b	1.91±0.52 ^a	0.42±0.25 ^a	0.97±0.14 ^a	1.94±0.18 ^b	0.69±0.28 ^b	0.97±0.14 ^a	0.56±0.14 ^a	0.96±0.27 ^a	0.54±0.12 ^a	11.83±0.6
20	1.66±0.13 ^b	1.53±0.42 ^b	1.96±0.53 ^a	1.37±0.27 ^a	0.46±0.27 ^a	1.37±0.28 ^b	1.51±0.13 ^b	1.22±0.41 ^a	0.77±0.41 ^a	1.06±0.40 ^a	0.45±0.01 ^a	13.35±0.5

s- Pollen mother cells, Sc- Scattering of chromosomes, Pm- Precocious movement of chromosomes, St- Stickiness of chromosomes, Un- Unoriented chromosomal sets, Lg- Laggards in chromosomes, Bg- Bridge formation in chromosomes, Dp- Dipolarity, Pr- Precocious Movement, Oth- Others, TAB% Total abnormality percentage (p<0.5)

Dose/ Biochemical components	Chl a	Chl b	Carotenoids
Control	6.95±0.05	3.83±0.04	1.42±0.02
20 Min.	6.97±0.07	4.05±0.09	1.47±0.01
40 Min.	7.84±0.08	4.51±0.08	1.49±0.02
60 Min.	7.58±0.03	4.19±0.06	1.53±0.02
80 Min.	6.63±0.09	3.65±0.09	1.59±0.01
100 Min.	5.85±0.03	3.37±0.06	1.68±0.01

Table 3: Data of biochemical components observed in the study

Biochemical analysis:

In case of biochemical results, the outcome is showing somewhat dramatically changing values. In case of *Chl a* and *Chl b*, the low exposure (40 min. & 60 min.) time proved beneficial and their level enhanced but at higher doses (80 min. and 100 min.) the concentration decreased. The data obtained in the study for both *chl a* and *chl b* were highest (7.84±0.08 and 4.51±0.08) at 40 min and lowest (5.85±0.03 and 3.37±0.06) at 100 min exposure. While in case of Carotenoids it was found that they are increasing with increasing exposure time of UV-B. The value of carotenoids content ranges from 1.42±0.02 in control to 1.68±0.01 in the plants treated with highest exposure time (100 min) (Table 3 and Fig 4).

These findings may be attributed to priming effect of UV-B radiations. In several studies the amount of photosynthetic and other pigments enhanced at low exposure of UV-B and UV-C radiation (Thomas and Puthur, 2020). Dwivedi *et al.* (2021) have also reported an enhancement at low exposure time in *Andrographis paniculata* and *Phyllanthus niruri*. While, at higher exposure duration, the declinment in the chlorophyll contents may be due to lipid peroxidation in chloroplast membrane (Rai and Agrawal, 2017). It can reduce the amount of chrolophyll as it targets some important enzymes of

photosynthesis (Erdieet *al.*, 2019). At high exposure time, it may suppress expression of some genes responsible for upregulation of photosynthetic pigments (Lizanaet *al.*, 2009).

Enhancement in the amount of carotenoid pigment in the experimental setup with increasing exposure time is due to photo-protective nature of carotenoids. The carotenoids are the pigments which generally encounter the stresses caused by different means to protect other photosynthetic pigments *i.e.*, *Chl a* and *Chl b* (Jaleel *et al.*, 2009)

Conclusion

From the present study, we can conclude that UV-B irradiation is beneficial at some extent but continuous exposure may lead to some disastrous outcome for the plant's growth and survival. Here in the study, we have clearly observed that initially the plant was rarely affected through the radiation (20 Min) and remains nearly similar to control one while at intermediate exposure (at 40 and 60 min) some of the beneficial characteristics were observed but at higher doses (80 and 100 Min) we can clearly see that the plants getting weak, underdeveloped and have shown stunted growth and poor vigour morphologically and biochemically. So, on behalf of the study, we can predict the use of UV-B irradiation in mutation breeding to develop more diverse plants of *Stevia* both morphologically and genetically and also conclude the effect of the long-term exposure of UV-B irradiation as the concentration of the same is increasing continuously in the environment due to continuously depleting phenomenon of ozone layer in stratosphere.

This study may lead to new horizons of the effect of UV-B radiation (which is continuously increasing in the environment due to continuous thinning of the stratospheric ozone layer) on the plant health including its cellular and biochemical health.

Declarations

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Authors Contribution:

Prof. Girjesh Kumar, planned the experiment, designed experimental setup and supervised the whole experiment and revised the manuscript critically.

Naveen Kumar Tiwari, performed all the experiment, collected the data and prepared the whole manuscript.

Satya Pandey worked in data interpretation, tables and graphs preparation and in manuscript designing.

Priyanka Pandey, arranged the references of the manuscript, formatted the Manuscript and revised it critically.

Jyoti Yadav, prepared the cytological table and plate, did final formatting and Data analysis of the manuscript.

All authors discussed the results and commented on the manuscript.

Statement of Data availability:

The authors confirm that the data supporting the findings of this study are available within the article. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

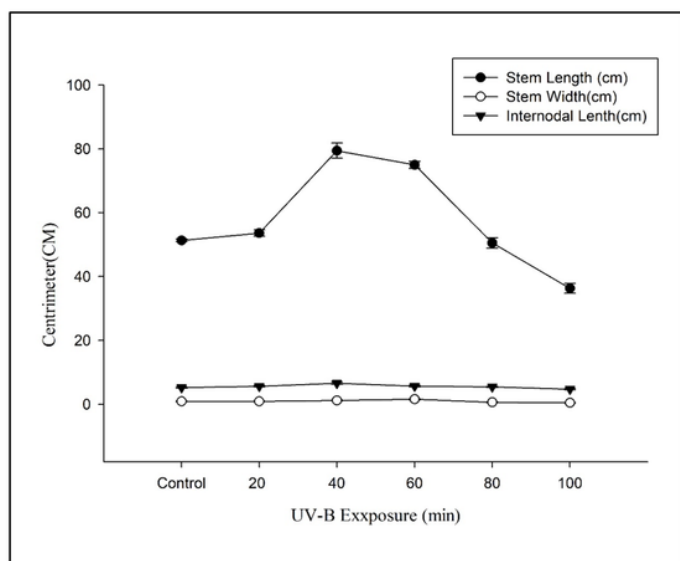


Figure 1(A): Graph representing different morphological parameters of stem

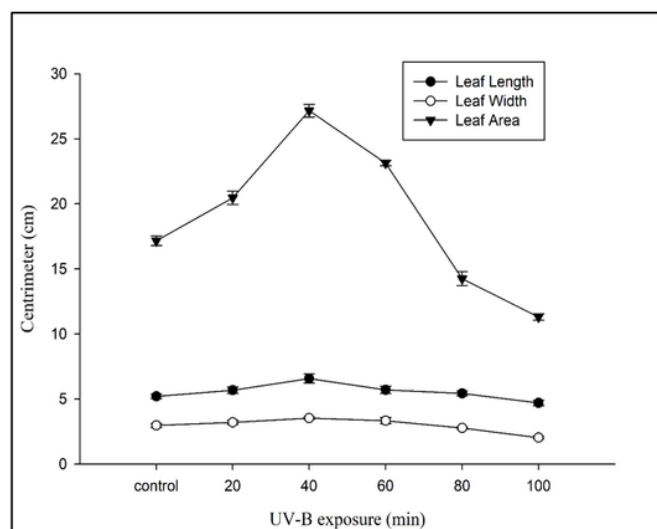


Figure 1(B): Graph representing different morphological parameters of leaf

Figure 1

See image above for figure legend.

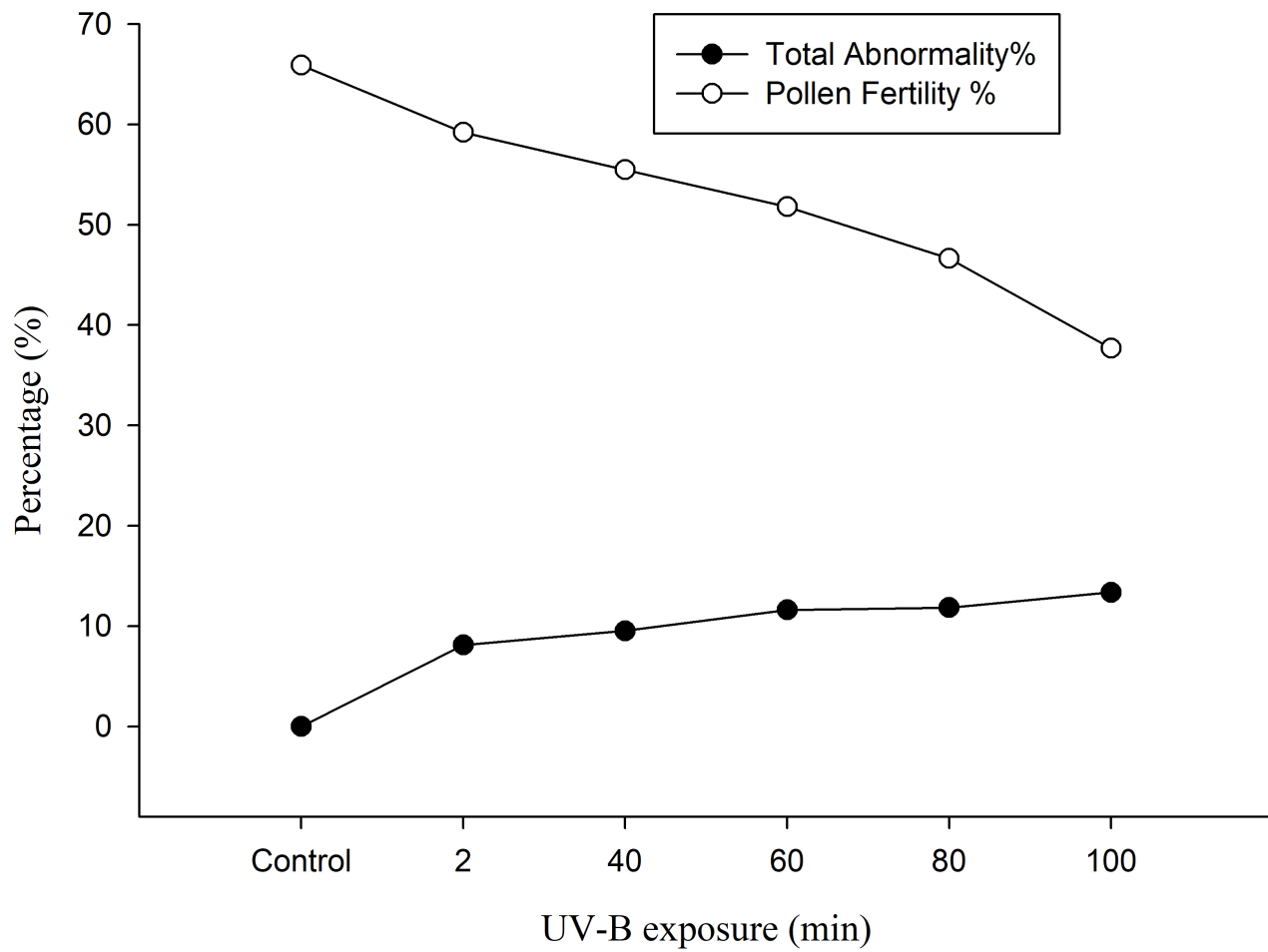


Figure 2

Pollen fertility and TAB% in UV treated set

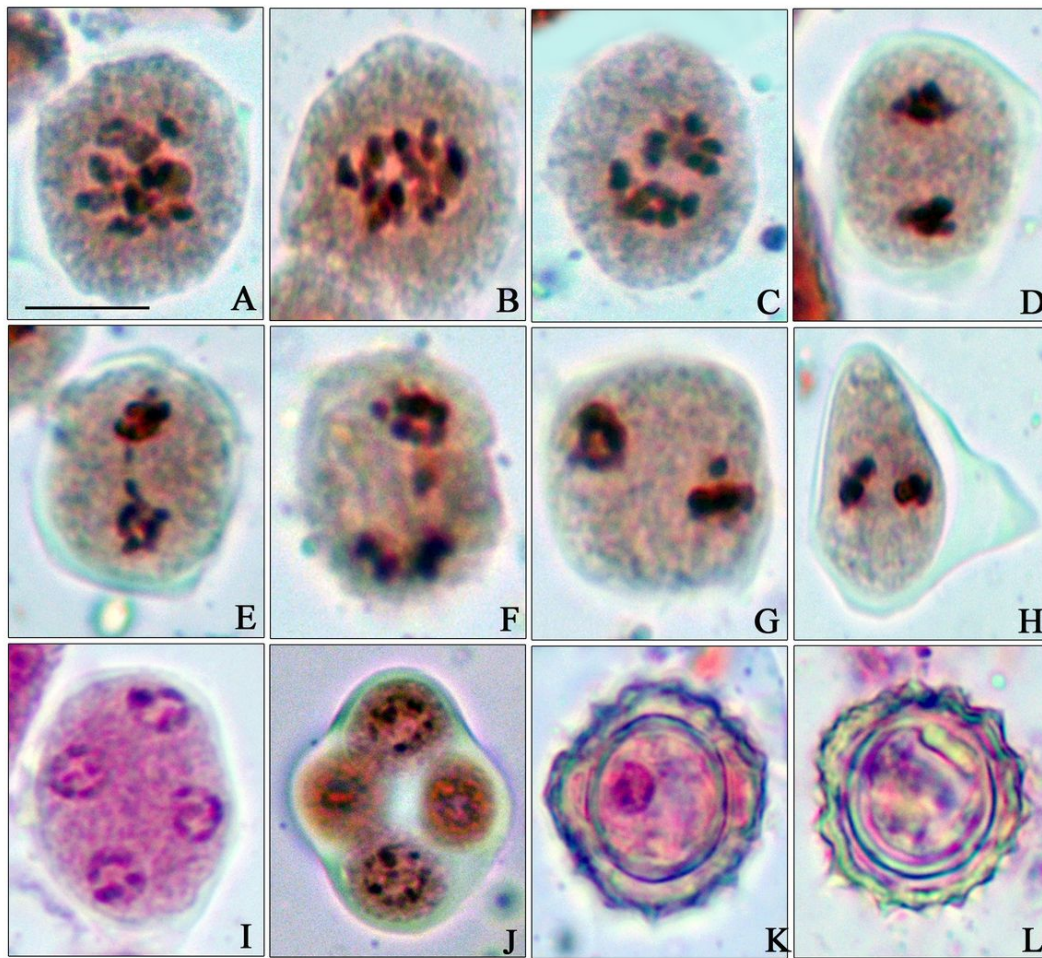


Figure 3

Cytological observations; A. Normal Diplotene, B. Normal Metaphase, C. Normal Anaphase, D. Stickiness at Anaphase, E. Bridge formation, F. Laggard, G. Precocious movement, H. Unorientation at Metaphase, I. Tetra polarity J. Normal Tetrad, K. Fertile Pollen, L. Sterile Pollen. (Scale- 5 μ m)

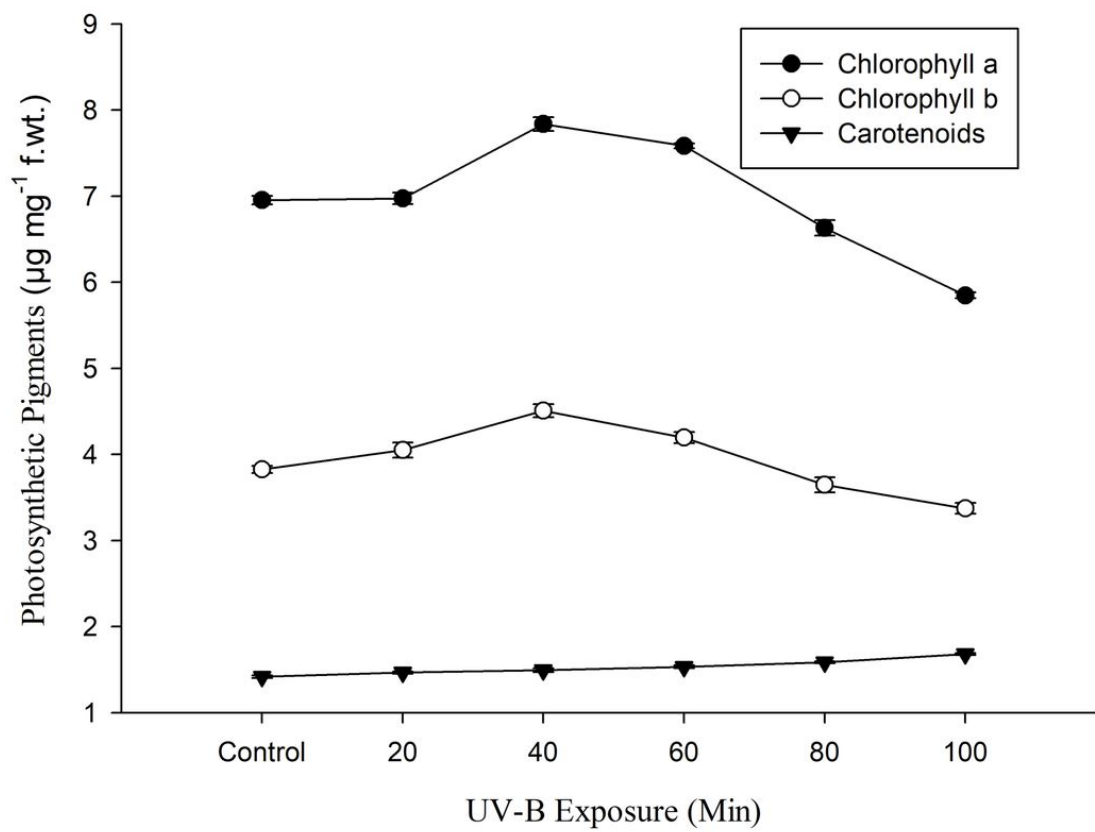


Figure 4

Graph representing the quantity of biochemical components observed during the study