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THE EFFECT OF LIGHT ON ANTHOCYANIN ACCUMULATION, ANTIOXIDANT CAPACITY, AND DNA METHYLATION IN *Capsicum annuum*

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Contents

1. INTODUCTION AND OBJECTIVES 3

2.LITERATURE REVIEW 5

2.1. Solanaceae 5

2.2. Morphology 5

2.2.1. Stem 5

2.2.2. Leaves: 6

2.2.3. Flowers: 7

2.2.4. Fruit: 7

2.3. Development of fruit colour in pepper (solanaceae) 8

2.4. BHLH and MYB transcription factors and WD40 repeat proteins 9

2.4.1. WD40 9

2.4.2 BHLH proteins 10

2.4.3 MYB proteins 10

2.5. Anthocyanin Biosynthesis: 11

2.5.1. Regulatory genes 11

2.5.2. Early Biosynthetic Genes (EBGs) 12

2.5.3. Late Biosynthetic Genes (LBGs) 13

2.6. Anthocyanin biosynthetic mechanism 13

2.7. Biological function of anthocyanins: 15

2.7.1. Antioxidant activity: 15

2.7.2. Benefits for plants: 16

2.8. Response of anthocyanin accumulation in *Capsicum annuum* based on environmental conditions 17

2.8.1. Effect of temperature on Anthocyanins 17

2.8.2. Effect of light on Anthocyanins 17

2.8.3. Health benefits for humans from anthocyanins 18
2.9. Postharvest fruit damage by pathogens 19
3. MATERIALS AND METHODS 20
3.1. Analytical measurements 20
3.1.1. TPC – Total Polyphenolic Content 20
3.1.2. FRAP (Ferric Reducing Antioxidant Power/Plasma) 21
3.1.3. TFC – Total Flavonoid content 22
3.2. Methylation pattern study 23
3.2.1. DNA isolation 23
3.2.2. DNA digestion and methylation 24
3.3. Detached leaf assay 25
4. RESULTS AND DISCUSSION 30
4.1. Analytical studies 30
4.1.1. Total polyphenolic content 30
4.1.2. FRAP (Ferric- reducing antioxidant power) 31
4.1.3. TFC (Total Flavonoid Content) 32
4.2. Methylation 33
4.3. detached leaf assay 35
4.3.1. NBT (Nitro Blue Tetrazolium) 35
4.3.2. DAB (3,3′-diaminobenzidine) 37
5. CONCLUSION 40
6. SUMMARY 41
7. ACKNOWLEDGMENT 42
8. BIBLIOGRAPHY 43

1. INTODUCTION AND OBJECTIVES

Pepper (*Capsicum* spp.) is a worldwide largely grown vegetable crop for its nutritional and economic importance. *Capsicum annuum, C. frutescens, C. chinense, C. baccatum* and *C. pubescens* are domesticated for consumption as food (Parisi et al., 2020). There are some purple pepper varieties which flowers, seeds, fruits and leaves contains anthocyanins a class of flavonoids that are soluble in water. Phenolic compounds are the primary class of antioxidant phytochemicals which acts as scavengers of free radicals (Abdelrahman et al., 2024). Anthocyanins (from Greek *anthos* – flower and *kyaneos* – blue) are plant low molecular weight compounds, defined as secondary metabolites, meaning pigments are not essential for the primary metabolism (Trojak & Skowron, n.d.). Anthocyanins are bioactive compounds and due to their natural antioxidant power, they can reduce the level of oxidative stress (Sadilova et al., 2006). Nasunin (delphinidin-3-p-coumaroylrutinoside-5-glucoside) is characterized in *Capsicum annuum* as one of the main anthocyanin derivatives. the purple ripening stage being a particular character of this variety (Sadilova et al., 2006). Anthocyanins are responsive to different environmental conditions, i.e.., the production of high levels of anthocyanin in young seedlings requires prolonged exposures to visible and near visible radiation at relatively high photon fluxes (Mancinelli, 1990). Better understanding of the anthocyanin function, synthesis and distribution as screening dyes may be crucial for selection of crop varieties characterized by increased anthocyanin accumulation. It may be beneficial as increased accumulation not only can enhance light-stress resistance but could also contribute to nutritional composition as well, as higher levels of anthocyanins can be translated as elevated number of polyphenolics in the plants which might result in an overall enhanced antioxidant capacity. Besides anthocyanin pigments are being effective against abiotic stressors such as light, several articles have already discussed their beneficial role against biotic stressors as well. For example, tomato studies showed that the anthocyanin accumulating transgenic lines acted better against *Botrytis cinerea* infection. Upon *Fusarium* infection purple grain wheats were more resistant to the infection and contained less mycotoxins than the anthocyanin-less varieties.

With this study the objective was **to evaluate the effect of light** on the polyphenolic pathway, by examining the covered and illuminated flowers of the same genotype, the genotype effect can also be ruled out. By covering the flowers, the aim was to examine the changes in terms of **how the different light conditions affect the total polyphenolic content, total flavonoid**

content and the overall antioxidant capacity next to the **methylation pattern changes** of the covered/illuminated flowers. Further, to test the **effectiveness of anthocyanin pigments against *Fusarium culmorum*.**

2.LITERATURE REVIEW

2.1. Solanaceae

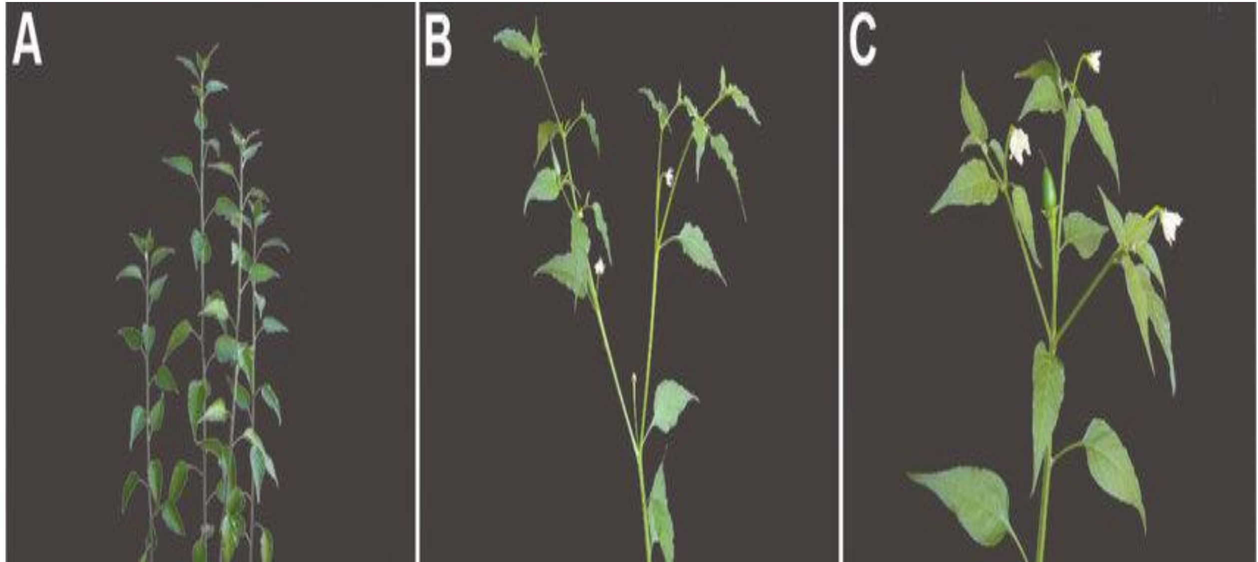
The Solanaceae family comprises 3000 to 4000 species which are classified in around 90 genera. However, there is a debate regarding this, as according to the D'Arcy in 1979, it estimated that Solanaceae family contains 83 genera and 2671 species, but the most recent estimate is that the family inclides more than 3000 species (Ganaie et al., 2018). The family includes ephemeral herbs (plants with a very short life cycle) like *Leptoglossis spp.* and *Schizanthus spp.* To large trees like *Duckeodendron* spp.*(Ganaie et al., 2018).* and occupies a wide range of terres trial habitats from deserts (eg. *Lycium barabrum*) to rainforests (eg. *Solanum sessiliflorum*) (Gebhardt, 2016), with a concentration of diversity in Australia and Latin America. From this large size of family only a few species in Solanaceae are considered to be important as food sources in human civilization such as *Solanum tuberosum, Solanum lycopersicum*, *Capsicum annuum*, *Solanum melongena*, *Solanum muricatum*, *Solanum quitoense* and *Solanum betaceum*. Few of them are classified as ornamentals eg. Petunia, Schizanthus. Plants from Solanaceae family also helps in producing drugs which are used for pharmaceutical uses are *Nicotiana tabacum*, *Atropa belladonna*, *Hyoscyamus*, *Datura alba, Withania somnifera, Mandragora* (Gebhardt, 2016b).

2.2. Morphology

2.2.1. Stem

Members of *Capsicum* plants are erect (eg. *C. schottienum),* compact (eg. *C. annuum var. annum)* or *postrate (eg.* C.annum var. glabruiusculum). They are subshurbs or shurbs, rarely trees (eg. *C.rhomboideum),* short lived perennials (eg. *C.chinensee)* or annual herbs (eg.*C. annum var. annum)*. Initial growth of the plant is monopodial first stem to emerge has 8-39 leaves. Before the onset of sympodial ramification and flowering alternate phyllotaxy emerges. The main stem ends in a solitary flower (fig 1) (Barboza et al., 2022a).

Figure 1. plant development in *C. chacoense* **A.** monopodial vegetative growth **B.** 1st dicotomy of the main stem and start of sympodial growth **C.** initial branching with three branches (Barboza et al., 2022b).



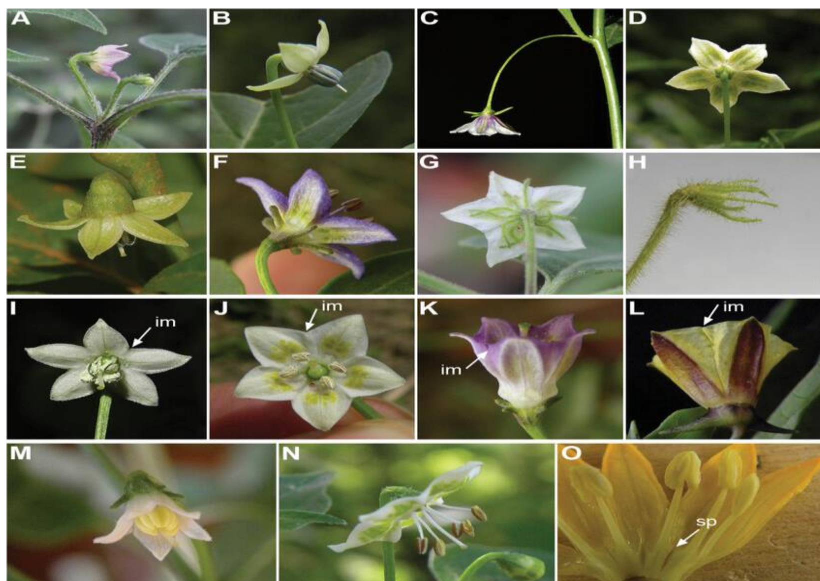
2.2.2. Leaves:

Capsicum species have simple leaves which are generally membranous or less frequently coriaceous (eg. *C.hunzikerianum, C. longifolium, C. pereirae).* Concolourous to discolourous, ovate or elliptical, rarely lanceolate or narrowly elliptical (*C. longifolium, C. carassense) in outline.* Leaf margines are always entire. Most of the species are amphistomatic (stomata present on both sides of the leaves) but there are some species which are hypostomatic (stomata on the lower side of the leaf) in *C. frutescens* and *C. annum, C. caatingae* etc. *(Barboza et al., 2022b).*

2.2.3. Flowers:

Pentamerous calyx and entirely synsepalous (sepals join partially). Calyx tube is campanulate and 5-10 nerved, with the margine always entire. Pentamerous corolla and sympetalous (fig 2).

Figure 2. flower morphology in *Capsicum* species **A** *C. rabenii* ***B*** *C. annuum var. glabriusculum* ***C*** *C. lanceolatum* **D** *C. schottianum* **E** *C. frutescens* **F** *C. eximium* **G** *C. eshbaughii* **H** *C. cornutum* ***I*** **C.** *galapagoense* **J** *C. recurvatum* **K** *C. cardenasii* **L** *C. lycianthoides* **M** *C. chacoense* **N** *C. baccatum var. baccatum* **O** *C. caballeroi. (Barboza et al., 2022b).*

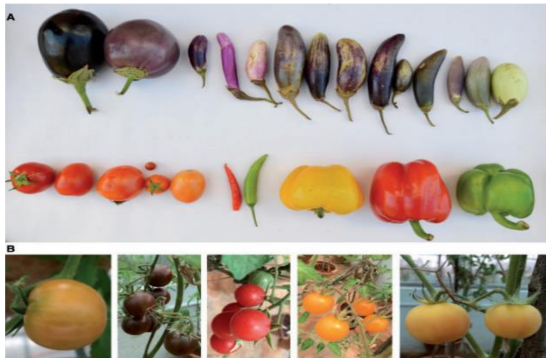


Medium size with 6-14 mm long and smaller size with 4-5mm long (Barboza et al., 2022a). The largest one reaches 17-1 8mm long (eg. *C. caballeroi, C. piuranum).* Flowers show different patterns of pigmentation (Carrizo García et al., 2016). Five stamens which are equal in length (Barboza et al., 2022a).

2.2.4. Fruit:

Bicarpellate berry that can also be 3-5 carpellate in domesticated species (eg. *C. annum var. annum, C. chinense).* The berries are extremely diversefied in size, shape and colour (fig 3).

Figure 3. fruit color diversity in A) brijal, pepper and tomato B) variation in fruit color in tomato (Dhar et al., 2015)



The greater variation of the fruit shape results in 100 names applied to the domesticated species. Given that there are three to ten pairs of heritable genes affecting fruit length with heritability value of 40-50 percent in capsicum (Zhigila et al., 2014) . Colours range from orange - red *(C. geminifolium*), dark burguny (*C. rhomboideumto*) dark purple (*C. regale*). Brazilian species have greenish-golden yellow translucent fruit (*C. parvifolium, C. schottianum).* Pericarp in capsicum is a true berry (Barboza et al., 2022a). Capsinoids in peppers frequently have a strong after taste particularly in pepper-based foods. The gene Pun1 is controlling the pungency in pepper identified as putative acyltransferase which catalyzes the last step of the alkaloid capsaicin biosynthesis (Gebhardt, 2016a). *Capsicum* fruits contain high number of vitamins like ascorbic acid (vitamin C), provitamin A, tocopherol (vitamin E), citrin (P), thiamine (B), riboflavin (B2), and niacin (B3) (Duranova et al., 2022).

2.3. Development of fruit colour in pepper (solanaceae)

Pigments in the plants are secondary metabolites which are organic compounds that are not essential for plant growth but provide additional advantages. Carotenoids and anthocyanins are mostly produced by large groups of plants mostly in solanaceae fruits to attract pollinators and forager to ensure pollination and seed dispersion and also provide advantages in stressful conditions. Anthocyanins and carotenoids belong to the two important clases of secondary metabolites, flavonoids (phenylpropanoid pathway) and isoprenoids (DOXP/MEP and MVA pathway) (Dhar et al., 2015).

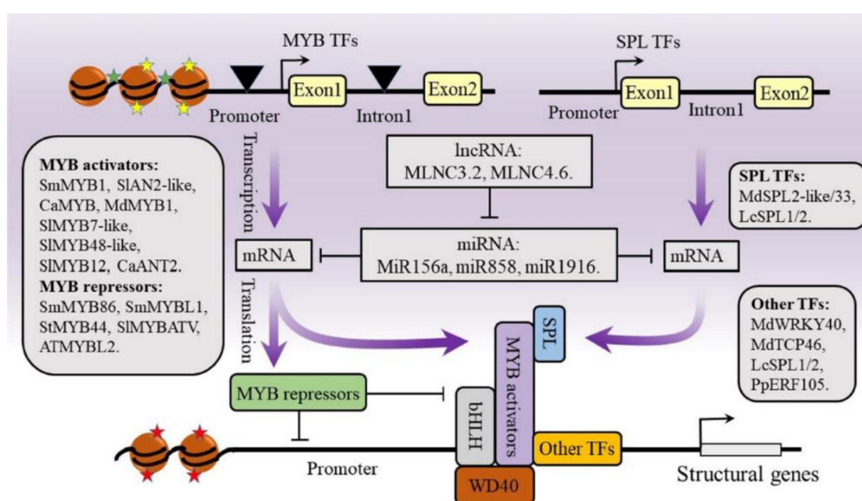
Anthocyanin biosynthesis is controlled by two types of genes one is anthocyanin biosynthetic structural gene and other is the regulatory gene which have three types of transcription factors

(TFs): MYB (v-myb avian myeloblastosis viral oncogene homolog) protein, BHLH (basic helix-loop-helix) protein, WD40 (tryptophan -W, aspartic acid- D) (WD-40 has a scaffolding function) protein. MYB (v-myb avian myeloblastosis viral oncogene homolog) transcription factors (TFs) occupy a dominant position in the regulatory network of anthocyanin biosynthesis (Aguilar-Barragán & Ochoa-Alejo, 2014).

2.4. BHLH and MYB transcription factors and WD40 repeat proteins

BHLH TFs occupies basic (B) region and helix-loop-helix domain (HLH). The basic region composed of 15-20 amino acids, and its function is to recognize E-box and G-box elements in the promoter of the target gene. HLH acquired for the formation of homo- or heterodimers of BHLH proteins locates at the C-terminus essential for the regulatory function. MYB proteins are a large family of transcription factors that regulate gene expression by binding to specific DNA sequence in target gene promotors (Dubos et al., 2010). WD40 is an indispensable transcription factor for anthocyanin biosynthesis. WD40 acts as a bridge to provide a platform for MYB and BHLH proteins to form a transcriptional complex (fig 4). In pepper plants whose *Ca*WD40 genes were silenced by virus induced gene silencing reduction in anthocyanin content was observed (Liu et al., 2018).

Figure 4. Transcriptional regulatory network of anthocyanin biosynthesis in plants (Li et al., 2022).



2.4.1. WD40

WD40 repeat proteins are the part of large family of β-propeller-shaped proteins. They contain repeating units called WD40 repeats each about 40 amino acids long delineated by a

glycine–histidine (GH) dipeptide and a tryptophan–aspartate (WD) dipeptide. These repeats fold together to form a propeller like structure. WD40 doesn't carry out chemical reaction instead act as a meeting point to bind with transcription factors like MYB and bHLH to form MBW complex. They influence visible traits like pigmentation eg. TTG1 in Arabidopsis, AN11 in petunia, PFWD in perilla (Yan et al., 2021). *Ca*WD40-91 interacts with *Ca*AN1(the bHLH) and *Ca*DYT1 complex in anthocyanin biosynthesis in peppers but causes male sterility (Tang et al., 2024).

2.4.2 BHLH proteins

The BHLH transcription factor represent one of the largest and most conserved families of regulatory proteins which plays a major role in controlling diverse biological processes such as development, secondary metabolism, particularly anthocyanin biosynthesis (B. Zhang et al., 2019). In basic helix-loop-helix the basic region binds to the DNA sequence at E-boxes. HLH region allows two bHLH proteins to join together and form dimers (Ramsay & Glover, 2005).

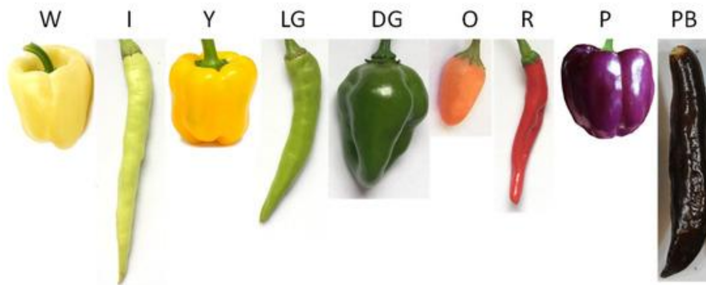
2.4.3 MYB proteins

MYBs contains a common binding domian (about 52 amino acids) that consists of 1-3 imperfect helix-turn-helix repeats abbreviated as R1, R2 and R3 are common in animals whereas R2R3-MYB are the most common in plants (Ramsay & Glover, 2005). In peppers several MYBs have been described already with relation to the anthocyanin biosynthetic pathway, out of which a novel study found that the expression of *Ca*MYB5 induced the expression level of anthocyanin biosynthesis structural genes and accumulation of delphinidin and cyanidin in peppers (H. Zhou et al., 2025).

Peppers and the petunia are the model plants to understand the complex anthocyanin biosynthetic pathway. Pigmentation is an evolutionary adaptation in which translocation and paracentric inversions have been major modes of genome evolution. Environmental conditions induced pigmentation which are normally not expressed before. Both anthocyanins and carotenoids share their precursors with other secondary metabolites like lignin, tannins and phytoalexins which are generated against environmental stresses. Such instances indicate that the nature of process determining the dominant pigmentation biosynthesis or degradation in plants (Khan et al., 2025). Studies proved that fruit color in Solanaceae has been affected during evolution by domestication (fig 5). Wild relatives of *Solanum melongena* stayed green until the half ripe, whereas cultivated varieties show

varieties of colorations due to absence or presence of anthocyanins and chlorophyll. Carbon dioxide supply from environment for anthocyanin biosynthesis also regulates the anthocyanin accumulation. Increase in carbon and UV-A supply promotes anthocyanin biosynthesis in shikimate pathway (Dhar et al., 2015).

Figure 5. Representative fruit color of pepper. W, White; I, Ivory; Y, Yellow; LG, Light Green; DG, Dark Green; O, Orange; R, Red; P, Purple; PB, Purple Black (Wang et al., 2023)



2.5. Anthocyanin Biosynthesis:

Expression of the regulatory and structural biosynthetic genes (EBGs and LBGs) is the primary level at which the induction or shut down of anthocyanin biosynthesis in plants is regulated (Y. Liu et al., 2018). EBGs encode enzymes that participate in the general flavonoid pathway these EBGs genes either express continuously or moderately influenced by the environmental conditions. LBGs enclose enzymes which catalyze the later steps of anthocyanin synthesis particularly these LBGs is highly responsive to environment conditions like light, temperature and pathogenic stress (Wang et al., 2014).

Flavanoid pathway produces different flavonoids along with the anthocyanins eg. Hydroxycinnamic acids, isoflavones, flavonols, phlobaphenes, pro-anthocyanidins and anthocyanins (Petroni & Tonelli, 2011).Anthocyanin biosynthesis involves 20 different organic molecules and 12 different catalytic enzymes encoded by multiple homologous genes (Meng et al., 2022).

2.5.1. Regulatory genes

Anthocyanin biosynthetic pathway is transcriptionally regulated by MBW complex.

MBW complex = MYB – b HLH -WD40 complex

The MYB transcription factors, together with the common BHLH and WD40 proteins, bind to the promoters of structural genes to largely define the activation or repression role of the

MBW complex (Y. Liu et al., 2018). MYB TFs play a major role in MBW complex due to their highly conserved DNA binding domain (MYB domain, consisting of 51–52 amino acids, represented by R). Based on the number of MYB domains, MYB transcription factors can be divided into three subfamilies: R3-MYB, R2R3-MYB and R1R2R3-MYB. Repressors consist of both R2R3-MYB and R3-MYB transcription factors (Li et al., 2022). Both types of MYB repressors can passively repress anthocyanin biosynthesis by competing with MYB activators for coupling to BHLH proteins in the MBW complex thereby reducing its activation capability. In addition, the R2R3-MYB repressors turn the function of the MBW complex from activation to repression through their repression motif which leads to active suppression of the transcription of downstream genes (Y. Liu et al., 2018).The expression of the *R2R3-MYB* and *bHLH* regulatory genes, is specific for pigmented tissue (Liu et al., 2018). In pepper, anthocyanin accumulation in fruits is determined by the activity of the *A loci*, which encodes *R2R3 MYB* (*CaMYB*) TF. Approximately 4 kb non-long terminal repeat (LINE-1 RETROTRANSPOSONS) inserting in the promoter or the first intron are essential for activating *CaMYB* expression in purple fruits. *Ca*ANT1 and *Ca*AN2 were the two additional MYB genes discovered which contributes to increasing anthocyanin content in peppers (Li et al., 2022). In peppers a MYC also known as CaAN1 plays major role in producing anthocyanin in leaves (Li et al., 2022). Transient VIGS (virus induced gene silencing) of $CaMYB_A$ effectively down-regulated the expression of both EBGs and LBGs and led to reduced anthocyanin content (Y. Liu et al., 2018).

2.5.2. Early Biosynthetic Genes (EBGs)

The particular structural genes produce the enzymes. These enzymes catalyze the first steps of the flavonoid pathway producing basic flavonoid skeletons like flavonones and dihydroflavonols they serve as the precursors for multiple brances of flavonoids. The enzymes which are produced from this EBGs are PAL (phenylalanine ammonia-lyase), C4H (Cinnamate 4-hydroxylase), 4CL (4-coumarate: CoA ligase), CHS (chalcone synthase), CHI (chalcone isomerase), F3H (Flavanone 3-hydroxylase). All this enzyme production is controlled by MYB activators (Meng et al., 2022). EBGs—*CHS, CHI*, and *F3H* are the common flavonoid pathway genes which are involved in the biosynthesis of all downstream flavonoids. *Ca*CHS upregulation is reported in anthocyanin pigmented fruits (*Capsicum annuum,* breeding line 06C59), while *Ca*CHA, *Ca*CHI and *Ca*F3H levels are expressed during the ripening of anthocyanin pigment in peppers (Aza-González et al., 2013).

2.5.3. Late Biosynthetic Genes (LBGs)

The enzymes produced from the LBGs catalyse the final flavonoid pathway which specifically results in anthocyanins. F3′H / F3′5′H – Hydroxylases that modify the flavonoid backbone, DFR – Dihydroflavonol 4-reductase, ANS (or LDOX) – Anthocyanidin synthase, UFGT – UDP-glucose: flavonoid 3-O-glucosyltransferase, OMT / GST – Methylation and transport enzymes for anthocyanin stabilization are the enzymes derived from the LBGs. All these enzymes production is activated by MBW complex (Petroni & Tonelli, 2011). LBGs—*F3'H, F3'5'H, DFR, ANS*, and *UFGT* are required for the biosynthesis of specific classes of flavonoids, including anthocyanins. In anthocyanin pigmented peppers during early fruit development *CaF3'5'H, CaDFR, CaANS*, and *CaUFGT* were upregulated and reaches maximum level until late unripe stage prior to ripening and later on gets downregulated which is corresponded to the transient anthocyanin accumulation pattern of these fruits (Wang et al., 2014).

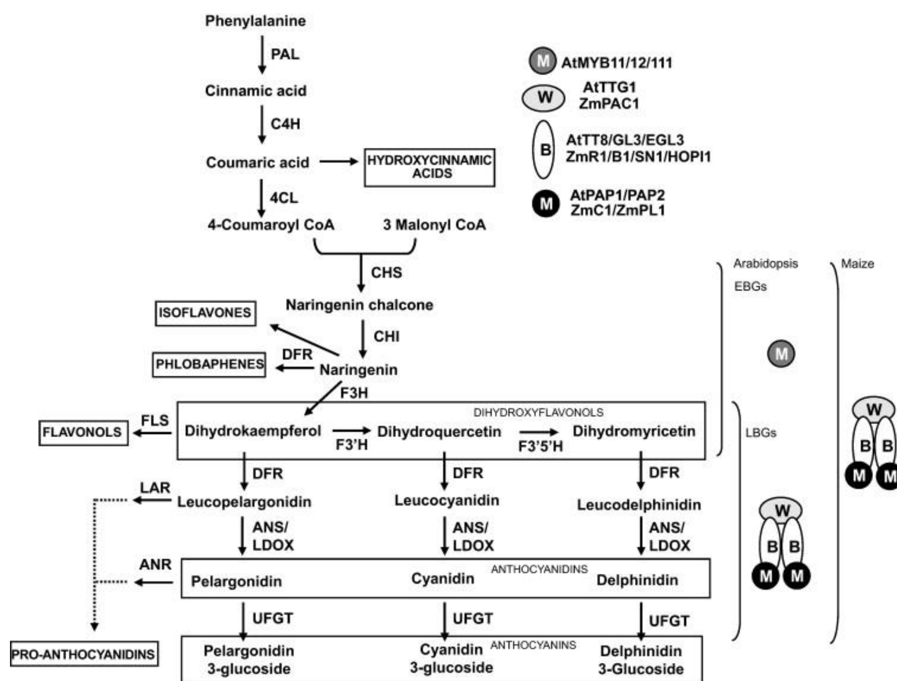
EBGs are expressed in sufficient levels in both pigmented and non-pigmented tissues because they are also responsible for producing not only for anthocyanins but also for other flavonoids. Whereas LBGs levels are significantly higher in pigmented tissues than non-pigmented tissues. Therefore, LBGs expression determines the quantitative variation of anthocyanins (Wang et al., 2014).

2.6. Anthocyanin biosynthetic mechanism

Anthocyanin biosynthetic pathway starts with the chalcone synthase (CHS) acts on 4-coumaroyl-CoA and malonyl-CoA and converts into naringenin chalcone. The Naringenin chalcone is isomerized by chalcone isomerase (CHI) to naringenin. Flavanone 3-hydroxylase (F3H) converts naringenin into dihydrokaempferol that can be further hydroxylated by flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′,5′-hydroxylase (F3′5′H) into two other dihydroflavonols, dihydroquercetin or dihydromyricetin. The three dihydroflavonols are converted into colorless leucoanthocyanidins by dihydroflavonol 4-reductase (DFR) and subsequently to colored anthocyanidins by anthocyanidin synthase (ANS) (fig 6). Finally, sugar molecules are attached to anthocyanidins by various members of the glycosyltransferase enzyme family, for instance, flavonoid 3-O-glucosyltransferase (UFGT), F3′H and F3′5′H are the primary enzymes responsible for the diversification of anthocyanins by determining their B-ring hydroxylation pattern and consequently their color (Y. Liu et al., 2018). Glutathione S-transferases (GSTs) and MATE transporters are the primary mediators

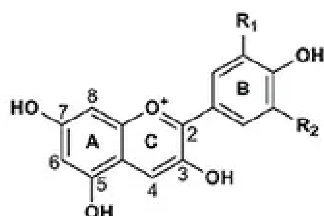
of anthocyanin trafficking and accumulation in vacuoles. GST sequesters anthocyanins from the cytoplasm into vacuoles in the first mechanism; transporters in the vacuolar membrane, such as ATP binding cassette (ABC) proteins and transporters, are essential in the second mechanism; and the fusion of the smaller vesicular structures with cytoplasmic anthocyanin bodies with central vacuoles is the basis for the final mechanism. Reactive oxygen species (ROS), superoxide radicals, hydroxyl radicals, alkoxy groups, hydrogenperoxide and singlet oxygens are constantly produced from mitochondria are indirectly removed by the GSTs to avoid oxidation of anthocyanins (Y. Zhou et al., 2022a).

Figure 6. Simplified scheme of flavonoid pathway, including phenylpropanoid pathway, the anthocyanin branch and other subgroups of flavonoid end-products (Petroni & Tonelli, 2011).



Anthocyanins are composed of anthocyanidin backbone with sugar and acyl conjugates. Anthocyanidins are composed of two aromatic benzene rings separated by an oxygenated heterocycle (fig 7). Out of 20 discovered anthocyanidins only 6 are prevalent in plants. They are pelargonidin, cyanidin, delphinidin are the primary anthocyanidins which differ each other by the number of hydroxyl group at B-ring, they show orange/red, red/magenta and violet/blue hues. Peonidin is derived from cyanidin by a single O-methylation at B-ring. Like wise petunidin and malvidin are derived from delphinidin O-methylation. Besides, the structure of anthocyanidin quantity and position of conjugated sugar and acyl moieties also leads to anthocyanin diversification (Liu et al., 2018).

Figure 7. General chemical structure of anthocyanidins and the six most common anthocyanidins in *Solanaceous* vegetables, indicated by “X” (Y. Liu et al., 2018).



Anthocyanidin	R_1	R_2	Pepper	Eggplant	Tomato	Potato
Pelargonidin (Pg)	H	H				X
Cyanidin (Cy)	OH	H				X
Delphinidin (Dp)	OH	OH	X	X	X	X
Peonidin (Pn)	OCH_3	H				X
Petunidin (Pt)	OH	OCH_3			X	X
Malvidin (Mv)	OCH_3	OCH_3			X	X

The main form of Anthocyanin in pepper fruits is delphinidin-3-(p-coumaroyl-rutinoside)-5-glucoside is a derivative of delphinidin identified in violet/black pepper. Acylated anthocyanins are the most abundant forms in pepper (Aza-González et al., 2012).

Change in pH from acidic to neutral in plant vacuole leads to reversible discoloration of anthocyanin molecules due to formation of colorless isoforms (Basílio & Pina, 2016). In Solanaceae discoloration in fruits begins to vanish when fruit reaches their maximum size it is because of the change in the balance between anthocyanin biosynthesis and degradation. There are some enzymatic and non-enzymatic factors that effect the anthocyanin concentration (Zhu et al., 2018). In purple pepper discoloration occurs due to decline in expression of positive regulatory genes and most of its downstream structural genes leading to decrease in anthocyanin biosynthesis (Liu et al., 2018). Vacuolar class III peroxidase, BcPrx01, was suggested to be the reason for anthocyanin degradation (Zhu et al., 2018).

2.7. Biological function of anthocyanins:

2.7.1. Antioxidant activity:

Free radicals, reactive oxygen species (ROS), reactive nitrogen species (RNS) are required for the cell signaling in low quantities if there are high ROS leads to the oxidative stress, cell damaging (Tena et al., 2020). Due to the presence of positively charged oxygen atom anthocyanins have a higher antioxidant capacity compared to other flavonoids. Hydroxylation at the B-ring enhances antioxidant capacity (−OH > −OCH3 >> −H), therefore the

antioxidant capacity of anthocyanidins decreases in the order of Delphinidin > Petunidin > Malvidin = Cyanidin > Peonidin > Pelargonidin. The more sugar units at C3 and C5 position, the lower the antioxidant activity (Y. Liu et al., 2018). Anthocyanins donate hydrogen atom or electrons from the hydroxyl group to ROS and converts them into non- reactive molecules. The resulting anthocyanin radical is stabilized by resonance in which it doesn't cause further damage (Tena et al., 2020).

2.7.2. Benefits for plants:

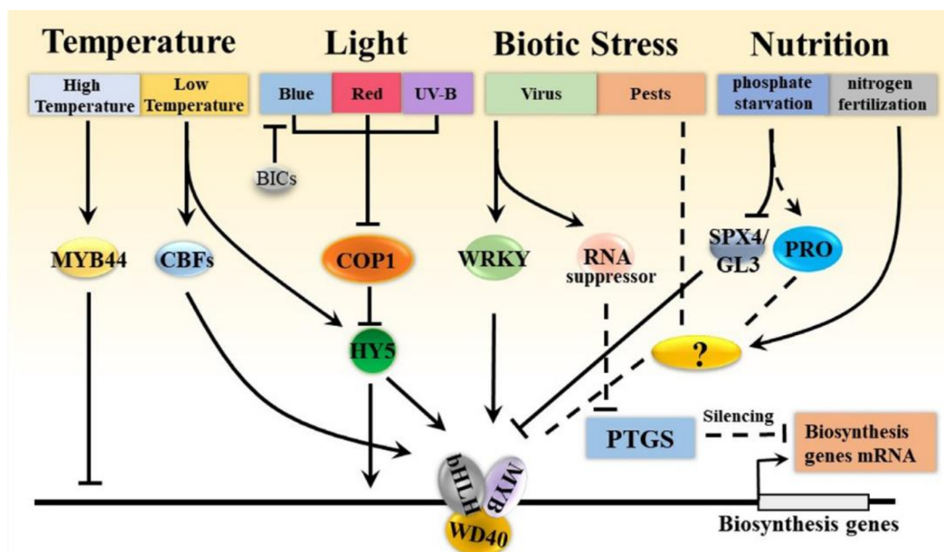
Anthocyanins protect the plants from severe abiotic and biotic stresses. Anthocyanins are photoprotective substances that scavange free radicals and absorb excess visible and UV radiation to shade and protect the photosynthetic apparatus. Purple pepper leaves rich in anthocyanins showed more stable PS II photosynthetic capacity and more photo-oxidation tolerance than non-anthocyanin tissues. Sun exposed side of the fruits, and young vegetative tissues frequently collect anthocyanins to shade them against photoinhibition and photobleaching in the presence of light stress without significantly compromising photosynthesis (Liu et al., 2018). Helps in pollination by attracting pollinators. Anthocyanins protect plants from severe biotic and abiotic stresses which helps in adopting to the environmental changes. Anthocyanins reduce the infestation of insects and pathogens. Anthocyanin can be used as visual markers for plant transformation and viral infection in biological research (Zhang et al., 2014).

The onset of anthocyanin accumulation is initiated with strong stimulation of the photosynthetic apparatus and the potential risk of phototoxic damage shifting the balance between capturing and utilization of energy mostly noted in aging or young developing tissues. It is postulated that anthocyanins allow restoring the redox balance and reducing the risk of oxidative damage as less energy is available for the photosynthetic apparatus (Steyn et al., 2002). Because they are photoinduced, researchers summarize that anthocyanins must have a photoprotective function, either against light-induced photo-oxidation or against UVB damage (Chalker-Scott, 1999). Anthocyanin rich plants show improved tolerance to low temperatures (Zhao et al., 2025). Fruits enriched with anthocyanins maintains cell integrity delay senescence and increase the self life (Liu et al., 2018). Anthocyanin accumulation is connected with the regulation of stress responsive genes for salinity and drought which is beyond pigmentation and antioxidant function (Ahmadzai et al., 2025).

2.8. Response of anthocyanin accumulation in *Capsicum annuum* based on environmental conditions

Accumulation and degradation of anthocyanins based on environmental conditions. High irradiance of UV/blue light and low temperature promotes anthocyanins biosynthesis. Whereas high temperature induces degradation of anthocyanins (Zhou et al., 2022). Numerous studies have recently revealed that viruses, light, temperature, and nutritional circumstances all affect the amount of anthocyanin that accumulates in Solanaceae fruits and vegetables (fig 8) (Li et al., 2022).

Figure 8. schematic representation of environmental factors regulating anthocyanin biosynthesis through MBW complex (Li et al., 2022).



2.8.1. Effect of temperature on Anthocyanins

Induction of anthocyanin at low temperature requires light. High temperatures cause plants to accumulate less anthocyanin by either increasing the expression of repressors or decreasing that of anthocyanin activators and related structural genes. The high temperature-dependent decrease in anthocyanin concentration was associated with an increased activity of a class III peroxidase and elevated H_2O_2 levels. Increased peroxidase activity at high temperature contributes in reduction of anthocyanins (Liu et al., 2018).

2.8.2. Effect of light on Anthocyanins

Light is the most influential environmental factor controls anthocyanin biosynthesis by activating transcriptional regulators (MBW complex) that induce pigment accumulation.

Light inactivates COP1- SPA complexes allowing HY5 (elongated hypocotyl 5) to accumulate and activate MBW complex this cascade reactions upregulates anthocyanin biosynthesis especially in high or UV light (Y. Ma et al., 2021).The study was conducted to explore the synthesis of anthocyanins in purple pepper under different light days showed positive results. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), 38 flavonoid metabolites were identified in the purple pepper germplasm HNUCA21, 30 of which are anthocyanins. Delphinidin-3-O-glucoside was detected in highest amount. Experiment on purple peppers under the light for 48 hours, 72 hours and 168 hours is observed by covering half of the fruit with bag shows the increase in anthocyanin content on the side of the fruit which is exposed to the light from 5 µg/g in 48 h to 43 µg/g in 168 h. the anthocyanin content on covered size remained constant between 0.9 and 1.8 µg/g in three time points (Y. Zhou et al., 2022a). High light mediated *Ca*MYB and *Ca*MYC expression in pepper, which in turn regulates *Ca*DFR and *Ca*ANS activity. Light causes *Ca*MYC to react continually, and the degree of its expression changes in direct proportion to the intensity of the light (Y. Zhou et al., 2022a). Blue and red light have been reported in inducing anthocyanin biosynthesis compared to darkness. Solanaceous R2R3-MYB activators such as *Sl*AN2 and *Ca*MYB_A, were upregulated by high light, whereas an R2R3-MYB repressor, *Ph*MYB27, was downregulated (Y. Liu et al., 2018).

2.8.3. Health benefits for humans from anthocyanins

Antioxidants capacity of anthocyanins Helps in decreasing chronic and degenerative diseases. The primary anthocyanins in solanaceous vegetables, delphinidin derivatives, have been linked to a decrease in vascular inflammation and a prevention of thrombosis. They may also protect the human skin from UV-B irradiance by inhibiting keratinocyte apoptosis. Capsicum species contain capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide) which cause the hot and spicy flavor. It is 'generally recognized as safe' (GRAS) as a food by the U.S. Food and Drug Administration (Bethesda, 2021). Capsaicin's interaction with Transient Receptor Potential Vanilloid 1 (TRPV1 receptors) supports its potential in pain relief, obesity control and cardiovascular health. Anthocyanins reduce inflammation and oxidative stress in humans (Ockermann et al., 2021). Some evidence suggests that anthocyanins improve brain health and protection against neurodegenerative conditions (Gonçalves et al., 2021). Recent studies showed that anthocyanins have prebiotic properties which helps in maintaining gut health by promoting proliferation of beneficial bacteria and eliminating harmful bacteria (Saini et al., 2024).

2.9. Postharvest fruit damage by pathogens

Twenty to twenty-five percent of harvested fruits and vegetables including peppers are impacted by rot-causing microorganisms during postharvest handling. In fruits and vegetables, fungal rots are mainly caused by *Alternaria alternata, Botrytis cinerea, Phoma* spp., *Geotrichum candidum, Fusarium acuminatum, Didymella lycopersici, and Stolonifer (Petrasch et al., 2019)*. The most dangerous pathogen, *A. alternata*, is more hostile to damaged fruits and weakens with extended storage. In fruits and vegetables, *A. alternata* typically causes brown to black patches that are followed by sunken lesions that spread out and occasionally appear on complete fruits. Self life is a quality trait for many fruits and vegetables; enrichment of anthocyanins can significantly increase the self life. Processes late in ripening are susceptible to *Botrytis cinerea* one of he most important post harvest pathogen is reduced in purple tomatoes and peppers (Zhang et al., 2013). The range of pathogens afflicting pepper is very broad and includes fungi (*Phytophthora capsici, Rhizoctonia solani, Verticillium dahliae, Colletotrichum scovillei* and *truncatum, Leveillula taurica, Fusarium* spp., bacteria (e.g., *Xanthomonas* spp.), viruses such as Tospoviruses (e.g., Tomato spotted wilt orthotospovirus and Impatiens necrotic spot orthotospovirus), Potyviruses (e.g., Potato virus Y, Tobacco etch virus, Pepper mottle virus), Tobamoviruses (e.g., Tobacco mosaic virus, Tomato mosaic virus), Cucumoviruses (e.g., Cucumber mosaic virus), nematodes (*Meloidogyne* spp.) (Parisi et al., 2020). *Capsicum annuum* exhibited more potent antibacterial activity than *Capsicum frutescens* extract and the analysis of phytochemicals detected existancs of polyphenols, flavonoids, alkaloids and sterols. Therefore *Capsicum spp.* Serves as natural antimicrobial properties (Abdelrahman et al., 2024).

Anthocyanins play an important role in postharvest protection. Acts as antioxidants, prevent lipid peroxidation and maintain membrane integrity to decrease cell senescence. Peppers enriched in anthocyanins showed less over ripening and a longer self life. At first anthocyanins increase the antioxidant capacity in fruits and vegetables, which supresses reactive oxygen species (ROS) activity and consequently delay the process of ripening. Secondly, anthocyanins increase fruit resistance to botrytis by altering the dynamics of the ROS burst generated by *Botrytis cinerea* infection, thereby limiting the induction of cell death required for growth and spreading of the fungus (Liu et al., 2018).

3. MATERIALS AND METHODS

The plants that I examined are from the F2 segregation from the cross between Fehérözön' a hungarian cultivar and a 'Black Pearl' a deep purple cultivar. I worked on generative parts especially the flowers. I covered at least two flower buds which are the first blooms with a thin silver foil to eliminate the illumination from five plants and exposed the whole plant to the sunlight (fig 9).

Figure 9. In all 1 to 5 plants two flowers are covered with silver foil to eliminate the illumination from five plants and remaining flowers are left uncovered.

After two weeks I collected both illuminated and covered flowers for further analytical and methylation pattern measures.

3.1. Analytical measurements

sample preparation:

Fresh samples were measured and ground using liquid nitrogen and ground using liquid nitrogen and mixed with a solvent mixture (60:39:1; 60 mL methanol, 39 mL distilled water, 1 mL formic acid) and subjected to centrifugation to separate the liquid and solid components. The supernatant was stored at -20°C for further analysis.

3.1.1. TPC – Total Polyphenolic Content

The TPC assay is used to measure all the phenolic compounds (flavonoids, phenolic acids, anthocyanins, etc.) by measuring its reducing capacity. In purple peppers the anthocyanin capacity is higher so the antioxidant capacity is also high which gives high TPC value (Choi et al., 2023). In the TPC we add Folin-Ciocalteu reagent which does not change or add any phenolic content to the sample but it acts as oxidizing agent. Folin-Ciocalteu contains

phosphomolybdic and phosphotungstic acids they react with compound which is capable of donating an elector or a hydrogen atom. Phenolic compounds including anthocyanins reduce the reagent by donating electron from its hydroxyl group (OH). Molybdenum (Mo^6+) and tungsten (W^6+) reduce to Mo^5+ and W^5+ during this reaction we get the blue color. Brighter the color which we measure with the spectrophotometer, more the reaction, higher the anthocyanin content. Gallic acid is a simple phenolic compound used as standard reference (like a bench mark) to measure the sample absorbance (Ribeiro et al., 2019).

The total polyphenolic content (TPC) was measured with Folin–Ciocalteu reagent according to Singleton and Rossi (1965), at $\lambda = 760$ nm with a Hitachi U-2900 spectrophotometer.

- The reaction mixture consisted of:
    - 50 mL Folin + 500 mL distilled water
    - 80:20 methanol: distilled water (MeOH: DW)
    - Na_2CO_3: 37.1 g in 500 mL DW
    - 3 mM Gallic acid: 0.056 g in 100 mL DW
        - 0,3 mM Gallic acid: 100 µl 3 mM Gallic acid in 900 µl MeOH: DW
- For the measurements 1250 µl Folin, 240 µl MeOH: DW (80:20) and 1000 µl Na_2CO_3 were measured into a test tube and 10 µl of the pepper extract was added. The samples were incubated at 50°C for 5 minutes. TPC was calculated based on the calibration curve of 0, 6, 12, 18, 24, and 30 µg/mL gallic acid, and the results are expressed as mg gallic acid equivalent (Ga)/g fresh weight.

3.1.2. FRAP (Ferric Reducing Antioxidant Power/Plasma)

Antioxidant Capacity Determination (FRAP Assay)

It is used to measure the antioxidants in the sample which reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions. Acetate buffer maintains the acidic condition to run the reactions. TPTZ (2,4,6-tripyridyl-s-triazine) (C16H12N6) it acts as a chelating agent to bind tightly to the metal ions here with Fe^{3+} metal ion. The ferric chloride is an unstable yellow solution which forms colorless complex with the TPTZ. TPTZ keeps iron ions stable at acidic condition otherwise they get precipitated. When the purple pepper flower sample is added to the Fe^{3+} TPTZ – complex, it turns blue because anthocyanins are the dominating phenolic compound which

reduce the Fe^{3+} to Fe^{2+} and form complex which is stable. $FeSO_4 \cdot 7H_2O$ is used to give the standard curve. More the FRAP value more the anthocyanin content (Apak et al., 2013).

To assess antioxidant capacity, a ferric-reducing ability of plasma (FRAP) assay was performed. The reaction mixture consisted of:

1. Acetate buffer (pH 3.6) – 0.9 g NaAc, 10 mL distilled water, and 1.6 mL glacial acetic acid.
2. TPTZ (2,4,6-tripyridyl-s-triazine) solution (40 µM) – 5 mL distilled water, 40 µL HCl (37%), and 0.031 g TPTZ diluted in 10 mL distilled water.
3. $FeCl_3 \cdot 6H_2O$ solution (20 µM) – 0.054 g $FeCl_3 \cdot 6H_2O$ in 10 mL distilled water.
4. $FeSO_4 \cdot 7H_2O$ solution – 0.0556 g in 100 mL distilled water.

A working solution (10:1:1; 90 mL acetate buffer, 9 mL TPTZ solution, 9 mL $FeCl_3 \cdot 6H_2O$ solution) was prepared and used for the measurements. Samples were measured in triplicates, 10 µL extract was applied for assay. The measurements were carried out using a Hitachi U-2900 spectrophotometer. The results are expressed in µmol ascorbic acid equivalent (As)/g fresh weight, calculated against the calibration curve.

3.1.3. TFC – Total Flavonoid content

In purple peppers anthocyanin biosynthesis is closely linked to the Total Flavonoid Biosynthesis because they follow the same biosynthesis pathway. $AlCl_3$ forms yellow colored metal flavonoid chelates complex. Al^{3+} chelates with the oxygen atoms of flavonoids -OH and C=O groups. $AlCl_3$ also chelates with other flavonoids in the samples because all the flavonoids have carbonyl and hydroxyl groups but in purple peppers flowers anthocyanins are more when compared to the other flavonoids so more the chelate complex more the anthocyanin content. Potassium acetate (CH_3COOK) maintains the basic environment which helps in enhancing the chelate complex and also prevents the aluminium precipitation. Quercetin was used to make calibration curve (CHIA-CHI CHANG, n.d.).

The total flavonoid content was determined by the aluminum chloride colorimetric method (Adefegha and Oboh, 2011). Accordingly, 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of potassium acetate and 2.8 ml of distilled water were added to 0.5 ml of supernatant. The absorbance was measured at λ = 415 nm using a Hitachi U-2900 spectrophotometer. The flavonoid content was calculated by comparing the calibration curve

of the quercetin standard (0, 20, 40, 80, 120, 160, 200 µg/ml), thus the results were expressed in mg quercetin equivalent per 1 g fresh weight.

3.2. Methylation pattern study

3.2.1. DNA isolation

Step 1- homogenize sample. Homogenize the plant material by using mortar and pestle by adding liquid nitrogen to obtain optimal yield.

Step 2- cell lysis. Transfer the resultant powder into an eppendorf tube and add 400 µL Buffer PL1. Alternatively, we can also use Buffer PL and vortex the mx thoroughly.

Step 3- RNase A. Add 10 µL RNase A solution and mix the sample thoroughly and then incubate the suspension for 15 min at 65 °C.

Step 4. 75 µL Buffer PL3, mix thoroughly and incubate for 5min on ice to precipitate SDS completely

Step 5- filtration/ clarification of crude lysate. Place a NucleoSpin® Filter (violet ring) into a new Collection Tube (2 mL) and load the lysate onto the column. Centrifuge for 2 min at 11,000 x g, collect the clear flow-through and discard the NucleoSpin® Filter. Alternatively centrifuge the crude lysate for 5min at 11,000xg and transfer the supernatant to a new tube.

Step 6- adjust DNA binding conditions. To adjust DNA binding condition 450 µL Buffer PC to the clear lysate and mix immediately by vertexing for 30s.

Step 7- bind DNA. Place a NucleoSpin® Plant II Column (green ring) into a new Collection Tube (2 mL) and load a maximum of 700 µL of the sample. Centrifuge for 1min and discard the flow through.

Step 8 (1st wash). Add 400 µL Buffer PW1 to the NucleoSpin® Plant II Midi Column. Centrifuge for 1 min at 11,000 x g and discard flow-through.

(2nd wash). Add 700 µL Buffer PW2 to the NucleoSpin® Plant II Midi Column. Centrifuge for 1 min at 11,000 x g and discard flow-through.

(3rd wash). Add another 200 µL Buffer PW2 to the NucleoSpin® Plant II Midi Column. Centrifuge for 2 min at 11,000 x g in order to remove wash buffer and dry the silica membrane completely.

<u>Step 9- elute DNA</u>. Place the NucleoSpin® Plant II Column into a new 1.5 mL microcentrifuge tube. Pipette 50 µL Buffer PE (65 °C) onto the membrane incubate it for 5min at 65 °C. centrifuge for 1min at 11,000 x g to elute the DNA. Repeat the step (GmbH, 2014)

3.2.2. DNA digestion and methylation

Methylation Sensitive Amplified Polymorphism (MSAP)-style protocol, where restriction enzymes and PCR are used to analyze DNA methylation patterns. The steps include digestion, ligation, and selective amplification.

MspI and HpaII recognize the same DNA sequence (CCGG) but differ in sensitivity to methylation. Comparing their digestion patterns reveals local DNA methylation. Hpal cuts only if the cytosine is unmethylated, stops cutting if cytosine is methylated whereas Mspll cuts through both methylated and unmethylated cytosine. By comparing fragments from MspII vs HpaI digestions, can decide whether the CCGG sites were methylated (fig 15). More the methylation lowers the genetic expression lowers the anthocyanin production.

EcoRI cuts at a different site, creating compatible sticky ends for ligation of adapters, enabling the amplification of specific DNA fragments.

In the first step, the samples were digested in separate reactions with the *Msp*II and *Hpa*I enzymes, followed by overnight incubation at 37 °C.

The digestion was performed in a total volume of 17 µl (containing 250 ng DNA, 1 µl of either *Msp*II or *Hpa*I, 2 µl of 10x Tango buffer, and distilled water). After incubation, the reactions were inactivated.

The following day, 1 µl of *Eco*RI enzyme and 2 µl of 10x Tango buffer were added to the mixture, which was again incubated overnight at 37 °C, followed by inactivation of the reaction. Adapters were then ligated to the digested DNA ends according to the Anza™ T4 Ligase Master Mix protocol.

Pre-selective PCR was performed using specific primers designed for the adapters. The resulting PCR product was diluted 50-fold.

The diluted sample served as the template for selective PCR, in which the primer pairs listed in the (Table 1) were used.

The separation of the resulting products was carried out on a 2% TBE agarose gel.

Table 1: Sequences of adapters, pre-selective, and selective primers

Adapter / Primer Name	Sequence 5'-3'
EcoRI – adapter – F	CTCGTAGACTGCGTACC
EcoRI – adapter – R	AATTGGTACGCAGTC
HpaII / MspI – adapter F	GATCATGAGTCCTGCT
HpaII / MspI – adapter R	CGAGCAGGACTCATGA
EcoRI – pre-selective	GACTGCGTACCAATTCA
HpaII / MspI – pre-selective	ATCATGAGTCCTGCTCGG
HpaII / MspI – TCAA	ATCATGAGTCCTGCTCGGTCAA
HpaII / MspI – TCAC	ATCATGAGTCCTGCTCGGTCAC
HpaII / MspI – GCT	ATCATGAGTCCTGCTCGGGCT
HpaII / MspI – CAG	ATCATGAGTCCTGCTCGGCAG
EcoRI – ACG	GACTGCGTACCAATTCACG
EcoRI – AAG	GACTGCGTACCAATTCAAG
EcoRI – ACA	GACTGCGTACCAATTCACA
EcoRI – AAC	GACTGCGTACCAATTCAAC
EcoRI – ACC	GACTGCGTACCAATTCACC
EcoRI – AAT	GACTGCGTACCAATTCAAT
EcoRI – ATC	GACTGCGTACCAATTCATC

3.3. Detached leaf assay

Helps to measure infestation on green leaves and purple leaves from same generation by using two staining methods one is DAB (3,3′-diaminobenzidine) in Detection of hydrogen peroxide during pathogenetic stress (Daudi et al., 2012). Another is NBT (Nitroblue

Tetrazolium) staining used to detect superoxide radicals during ROS stress (Venisse et al., 2001).

The detached leaf assay was done on the same F2 plants. For the study green and purple leaves were taken off from the plants and sterilized in sodium hypochlorite for 20 minutes. The sterilized leaves were then placed onto Murashige and Skoog media under sterile conditions in a laminar air flow. The inoculation of the leaves was done with the agar slice method. For this 10 days old *Fusarium culmorum* cultures grown on potato dextrose agar (PDA) media were used, agar slices were cut out from the *Fusarium plates* with a 5 mm wide sterilized cork borer and placed onto the adaxial side of the leaves directly on the main vein. The leaves were then incubated with the agar slices for 6 days (fig.10, 11). For the control, purple and green leaves were used to which sterile PDA slices were put. Along the 4 days incubation leaves were photographed daily and the images were processed with Image J software, to calculate the percentage of infection (fig. 10, 11).

Figure 10. inoculation of *Fusarium culmorum* on green leaves and observed for 4 days.

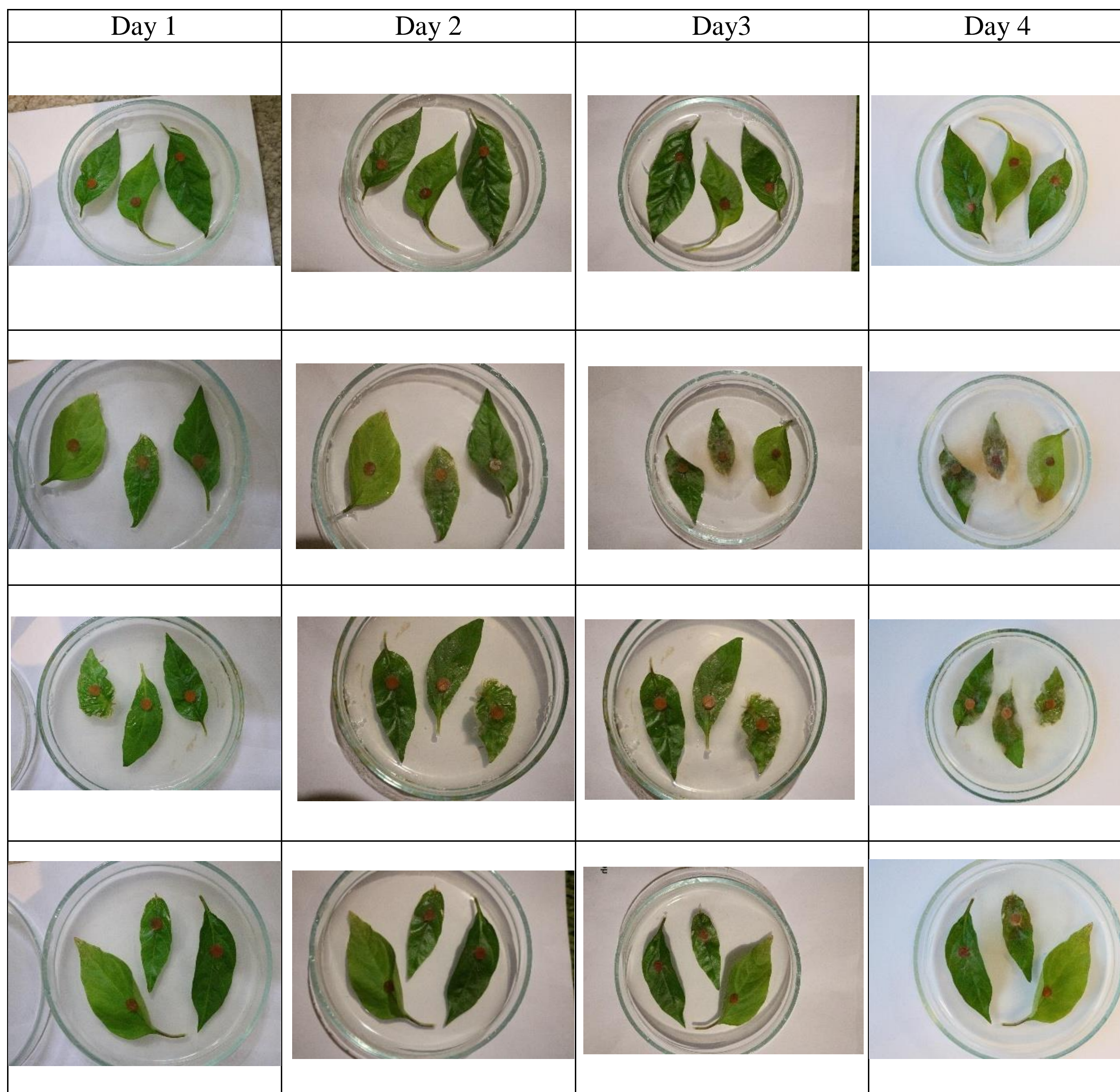
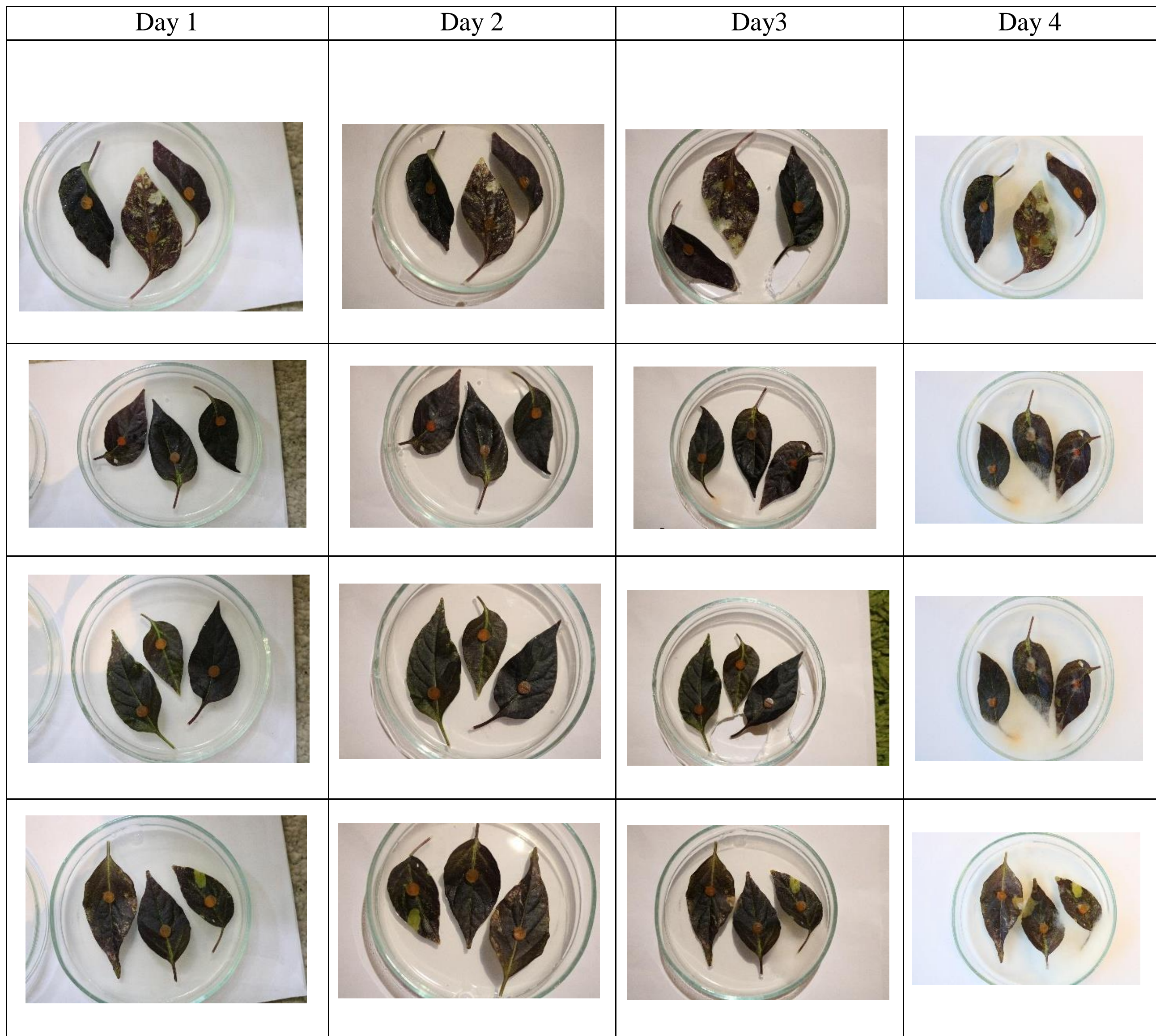


Figure 11. inoculation of *Fusarium culmorum* on leaves purple and observed for 4 days.



After 4 days the histochemical detection of H_2O_2 and $O_2^{\bullet -}$ was also performed. For H_2O_2 detection, leaves were immersed in 10 mL of a 1 mg/mL 3,3′-diaminobenzidine (DAB) staining solution (pH 3.8). For superoxide anion ($O_2^{\bullet -}$) detection, leaves were immersed in 10 mL of a 0.2% (w/v) nitro blue tetrazolium chloride (NBT) staining solution (pH 7.5). Samples were incubated overnight at room temperature in the dark. The following day, 10 mL of absolute ethanol per sample was added to the leaves, which were then placed in a 90C water bath for 10 min to remove chlorophyll. Finally, the chlorophyll-free leaves were fixed in 5 mL per sample of 60% glycerol. Images were captured and analyzed with Image J.

The superoxide dismutase (SOD) as well as the peroxidase (POD) enzymatic activity was also measured.

For the POD activity measurement samples were mixed with a buffer containing 8 mM guaiacol and 100 mM sodium phosphate pH 6.4. After the addition of 24 mM H_2O_2 as a substrate, the change in the absorbance was recorded at λ = 460 nm in 60 s intervals with a Hitachi U-2900 spectrophotometer. As for the SOD the reaction mixture contained 50 mM sodium phosphate buffer, 10 µM EDTA, 13 mM L-methionine, 75 µM nitroblue tetrazolium (NBT) and 2 µM riboflavin. During the reaction assay preparation, the mixture was kept in dark and to kickstart the reaction, the ready reaction mixture was illuminated with luminescent light for 10 min. Absorbance was measured at λ = 560 nm wavelength

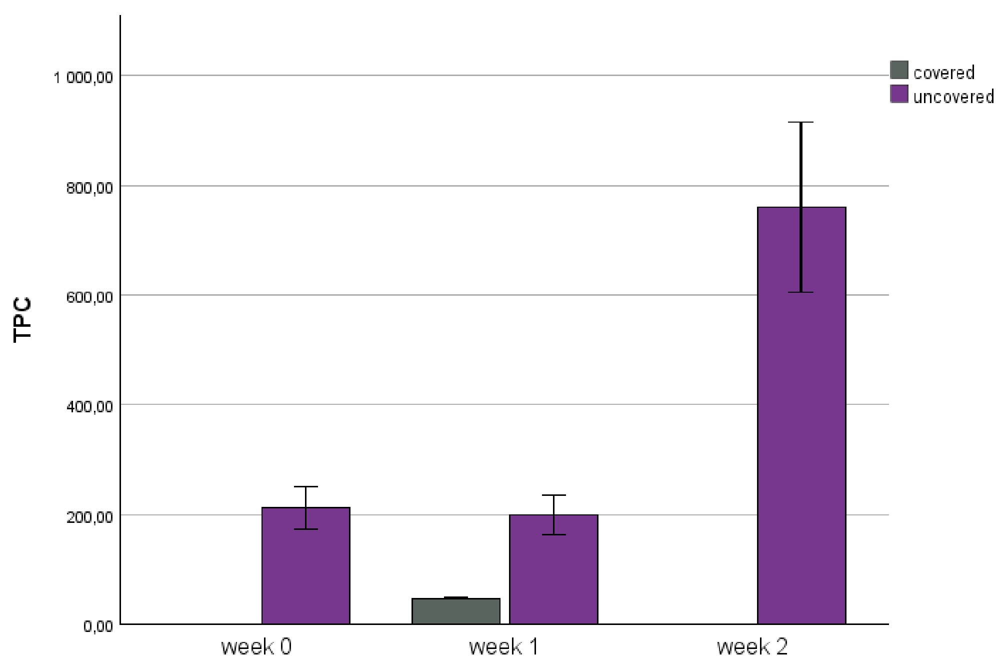
4. RESULTS AND DISCUSSION

4.1. Analytical studies

4.1.1. Total polyphenolic content

TPC assay is used to measure all the phenolic compounds present in the plant extract. TPC for the covered and non covered *Capsicum annuum* flowers was determined using the Folin-Ciocalteu to evaluate the effect of light exposure on the accumulation of phenolic compounds. Since phenolics, particularly anthocyanins contribute significantly to the antioxidant potential and coloration of the purple peppers. The assay measures the overall reducing capacity of phenolic constituents expressed as gallic acid equivalents (mg GAE/g FW) allowing a quantitative assessment of total phenolic concentration.

Figure 12. TPC of week 0, uncovered and covered week 1 and week 2 plants



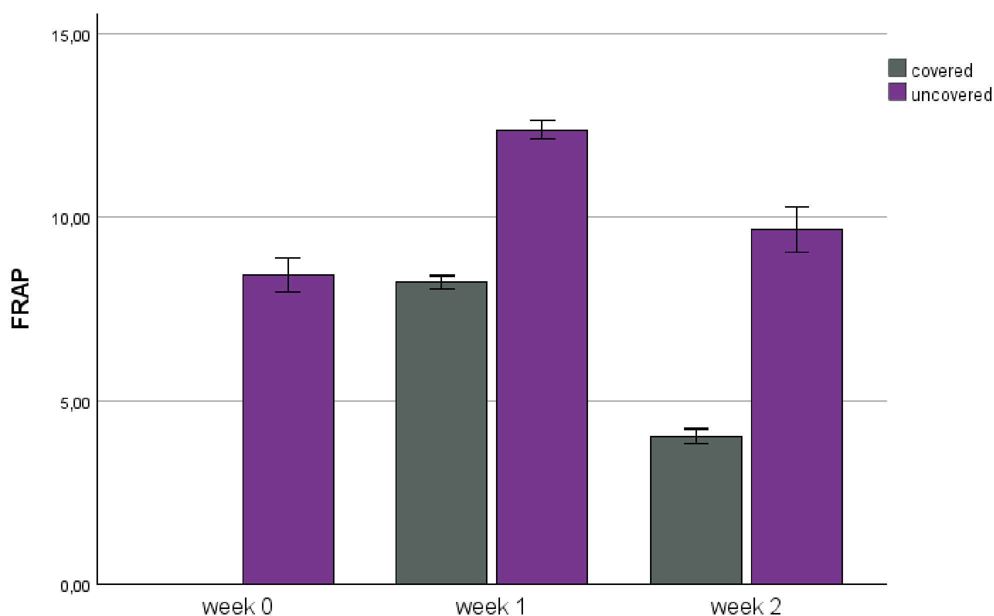
Based on the results from (fig 12) the polyphenolic content increased from 211 gallic acid equivalent from the zero week uncovered flowers to 760 gallic acid equivalents in the 2nd week flowers. It explains that the phenolic content increases with increase in the exposure of the light where as in purple peppers anthocyanins are dominant. According to the article (Chalker-Scott, 1999) it is also mentioned that phenolic content increases with light exposure.

From another source mentioned that the TPC value increases with modification of light (Jiménez-Viveros et al., 2023).

4.1.2. FRAP (Ferric- reducing antioxidant power)

The antioxidant potential of both covered and uncovered *Capsicum annuum* purple flowers has been evaluated based on FRAP. The phenolic compounds especially the anthocyanins which are abundant in the sample reduce the ferric ions to ferrous ions by donating electron. When the Fe^{3+} TPTZ changes to Fe^{2+} TPTZ the sample changes to blue color which indicates the reduction of ferrious ions by antioxidants, depper the color more the antioxidant capacity. The FRAP measures are expressed as µmol ascorbic acid equivalents per gram of fresh weight (µmol As /g FW).

Figure 13. The FRAP value in week 0, week 1 and week 2 covered and uncovered flowers



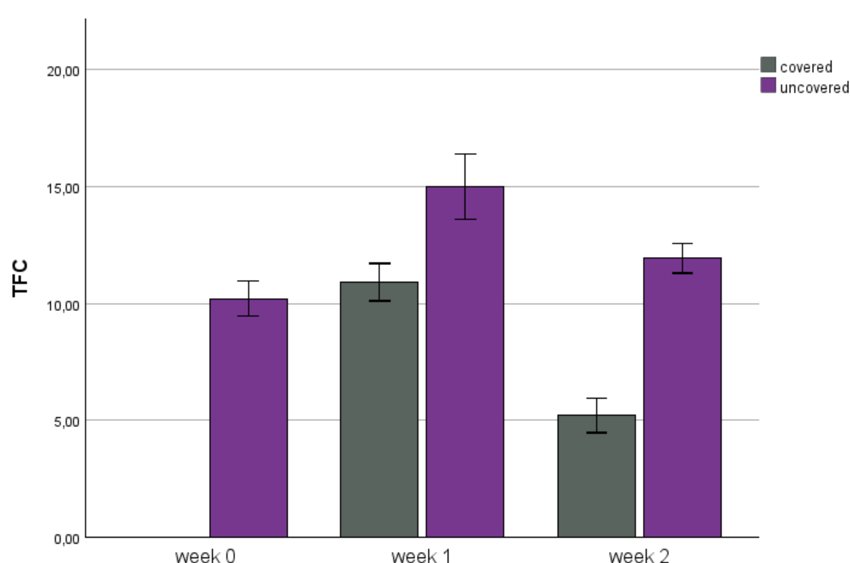
Week-1 uncovered samples exceeds both 0-week and 2nd-week uncovered samples. 2nd week's uncovered value is around 10 µmol As /g FW where as 1st weeks uncovered average value is 27 µmol As /g FW for the zero week it is around 9 µmol As /g FW. The covered leaves showed the lower value 1-week it is 8 µmol As /g FW and in the 2nd -week it is 4 µmol As /g FW (fig 13) explains lower antioxidant capacity. Initially light stimulates flavonoid biosynthesis boosting reducing power measurable by FRAP. In peppers such light induced

anthocyanin accumulation has been demonstrated under varying light durations like anthocyanin levels peaked at ~168 h of light treatment (Y. Zhou et al., 2022b). Due to physiological and biochemical changes anthocyanin synthesis may slow down. Second week suggests that while light exposure initially stimulates antioxidant production extended exposure may cause pigment degradation (Enaru et al., 2021). From all these observations it is clear that light plays a crucial role in phenolic compounds biosynthesis.

4.1.3. TFC (Total Flavonoid Content)

This method gives the data of all the flavonoids in the sample whereas in purple pepper flowers the dominante flavonoid is anthocyanin so it is obvious to get to know the dominant flavonoid content in the samples. Here I am examing covered and uncovered flowers so it is possible to sentence how light exposure influence the synthesis of flavonoid compounds. In this method aluminium chloride chelates with hydroxyl and carbonyl groups of flavonoids gives yellow colored chelate complex. More the chelation, depper the yellow color result of more flavonoid content. The resulting color samples are measured at 415 nm spectrophotometrically. Quercetin is used as a standard to generate calibration curve and results were expressed as mg QE/g FW (fresh weight).

Figure 14. TFC of the week 0, week 1 and week 2 covered and uncovered flowers



From the graph we can observe that 1st week uncovered flowers have more value which is around 14.7 QE/g FW where as 0-week and 2nd-week uncovered flowers have 10 QE/g FW

and 11.9 QE/g FW. Whereas 1st week and second week covered flowers have 11 QE/g FW and 11.9 11 QE/g FW (fig 14). I have observed that there is slight higher value for the 1st week covered flowers to the 0-week uncovered. There is a high chance 'Al' might chelate with with other dominant flavonoids (CHIA-CHI CHANG, n.d.). So according to the article there is a high chance that Al^{3+} might chelate with the other flavonoids in the samples. So, if there are more flavonoids in week-1 sample than the week-0 irrespective of illumination of the flowers, because not all flavonoids are light induced like anthocyanins there might be a change in expected difference among covered and uncovered flowers. In the 1st week uncovered flowers the TFC value is more the sample we used is purple flower so there is high probability of chelate formation with anthocyanins. Covered samples from 2nd week have low TPC values due to no light to do flavonoid synthesis. Over all we can say that TFC value can be high for uncovered.

4.2. Methylation

Increase in BONSAI methylation 1 (IBM1) plays a crucial role in anthocyanin accumulation induced by High Light (HL). Increase in IBM1 leads to demethylation of SPA (SUPRESSOR OF PHYA) chromatin (decrease DNA methylation, decrease Demethylation of histone H3 lysine 9 (H3K9me2) allows SPA transcription results in anthocyanin synthesis. Loss of IBM1 leads to hyper methylated SPA reduce the SPA expression leads to uncontrolled anthocyanin buildup (Fan et al., 2024). DNA methylation in the MdMYB1 promoter directly controls the anthocyanin biosynthesis in granny smith and golden delicious apple cultivars. When both the varieties are bagged and re-exposed to the sun, they developed red or pink pigmentation, light exposure reduced methylation in the promoter and activates MdMYB1 expression leads to anthocyanin production. Treating with 5-aza-2′-deoxycytidine (5-aza-dC) a DNA demethylation agent triggers the anthocyanin biosynthesis (Ma et al., 2018). This information gave the base to study how methylation effects anthocyanin pigmentation in peppers.

Figure 15. methylation band patterns

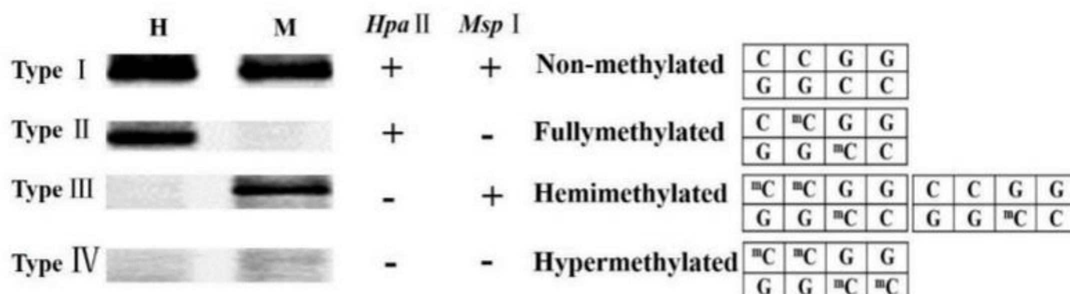
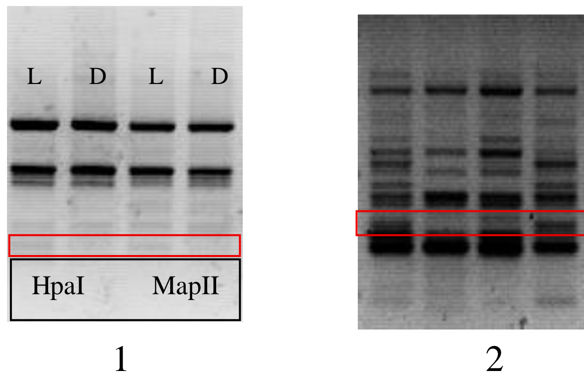


Figure 16. In the above gel images (1 and 2) we observed the function Of HpaI and MspII in pepper flowers who are in light and dark phases. L and D represent light and dark.



From figure 16/1- HpaII and MspI are both negative in dark and both are positive in light of the strands which are marked red. Which means there is hypermethylation at dark and non-methylated at light. Lower the methylation higher the anthocyanin biosynthesis. By that we can assume that their anthocyanin production is higher at light.

From figure 16/2- HpaII and MspI in light it showed '+' and '- 'whereas in dark it showed '- 'and '+' of the strands which are marked red. Which means in light it is fully methylated and in dark it is hemi- methylated. So, there is low anthocyanin production from both the phases due to methylation. The differences observed between the covered (dark) and illuminated (light) segments methylation are encompassed in table 2.

Table 2. Methylation patterns in light and dark phases.

Type	L		Difference	D		Pattern difference between L and D
	H	M		H	M	
I	+	+	I1	-	+	0
			I2	+	-	1
			I3	-	-	3
II	+	-	II1	+	+	4
			II2	-	-	3
			II3	-	+	2
III	-	+	III1	+	+	2
			III2	-	-	4
			III3	+	-	1
IV	-	-	IV1	+	+	5
			IV2	+	-	0
			IV3	-	+	1

To understand the epigenetic regulation on anthocyanin biosynthesis we compared the methylation patterns in dark (D) and light (L) phases. There are 4 methylation band patterns which explains whether the CCGG genes are non-methylated, fully methylated,

hemimethylated and hypermethylated (fig 15). These methylation patterns differes form Light to Dark samples during comparision which explains that methylation varies for the samples which are exposed and which are isolated from the sun light. Similar kind of study was conducted on red pear says that bagged (dark phase) pears showed high methylation. When the bag was removed there was a notable decrease in methylation (H. N. Liu et al., 2023). Differently methylated patterns in light and dark phases are associated with anthocyanin biosynthesis. This explains that light leads to less methylation and promotes anthocyanin biosynthesis, whereas the darkness might induce methylation and restrict anthocyanin biosynthesis.

4.3. detached leaf assay

4.3.1. NBT (Nitro Blue Tetrazolium)

Superoxide radicals (O_2^-) which is an early oxidative burst reduce NBT (yellow dye) to blue insoluble formazan crystals**.** The blue coloration indicates superoxide accumulation site around the infection zone in green leaves (fig 17) and in purple leaves (fig 18). Deeper the color and color spreading area indicates the severity of the infestation. Observing whether the antioxidants in purple leaves scavenge the superoxide radicals to reduce infestation.

Figure 17. NBT stained green leaves which are infected with *Fusarium*

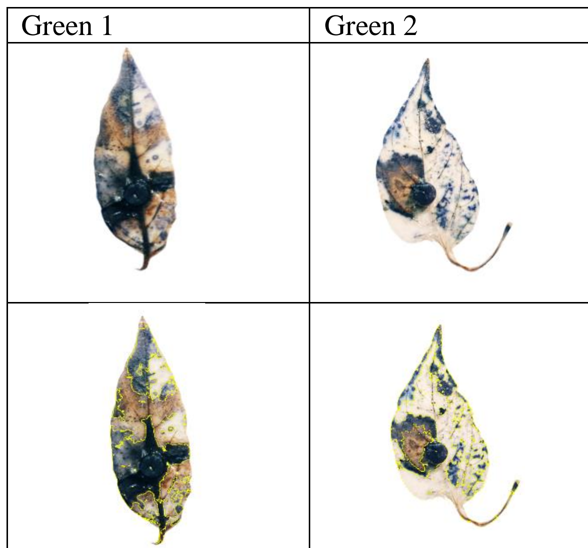


Figure 18. NBT stained purple leaves which are infected with *Fusarium*

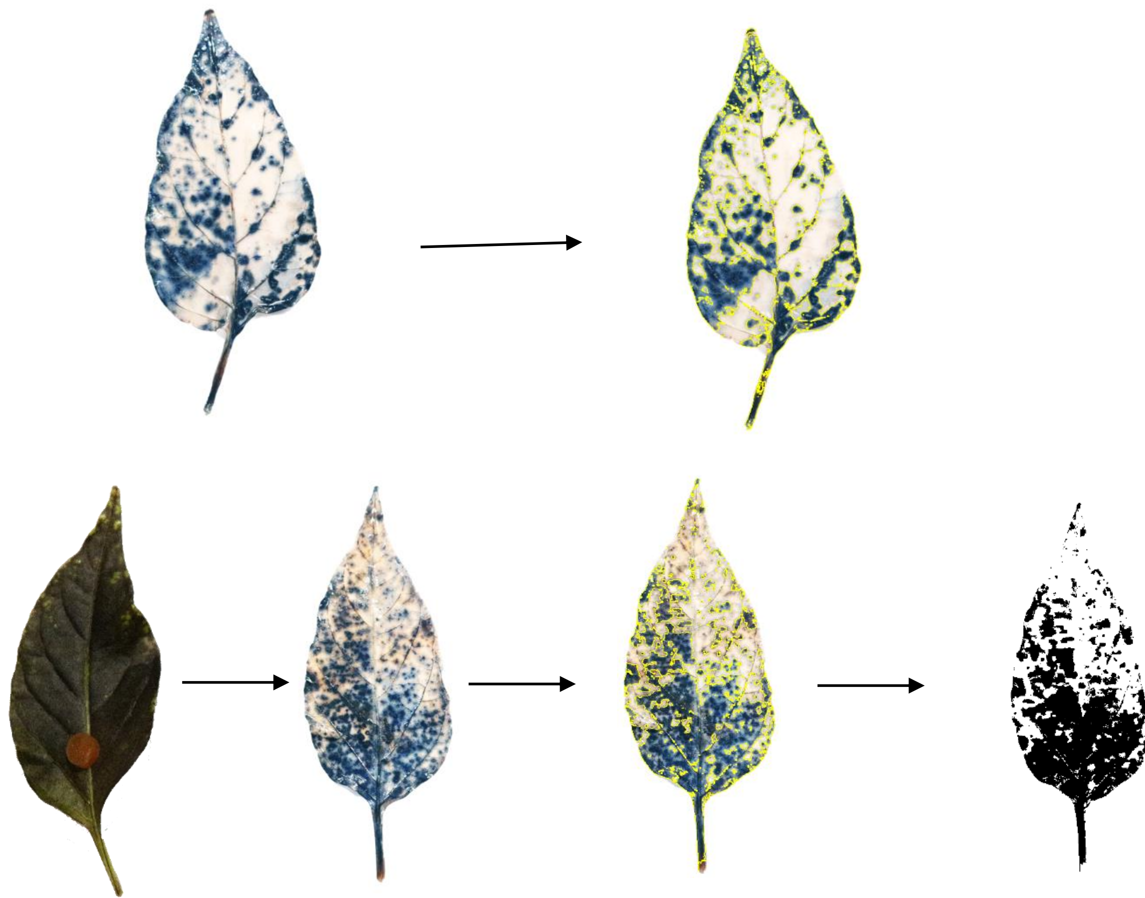
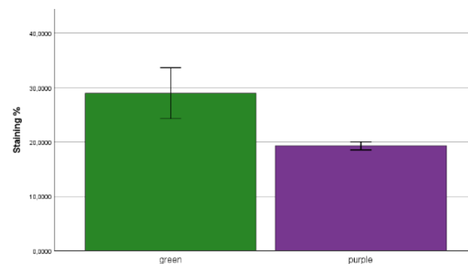


Figure 19. NBT staining percentage (1) and graphical (2) representation in green and purple leaves

Staining %	Leaf color
45,51662	Purple 1
53,59357	Purple2
45,00863	Purple3
50,61629	Green1
36,80456	Green 2
37,08634	Green 3

1



2

In both graphical and numerical values shows that purple leaves show lower NBT staining (fig 19/1 and 2). which means lower ROS levels, better oxidative balance due to higher antioxidant protection from anthocyanins. Green leaves show the higher NBT staining (fig 19/1 and 2). This is because lack of oxidative balance and low anthocyanin content.

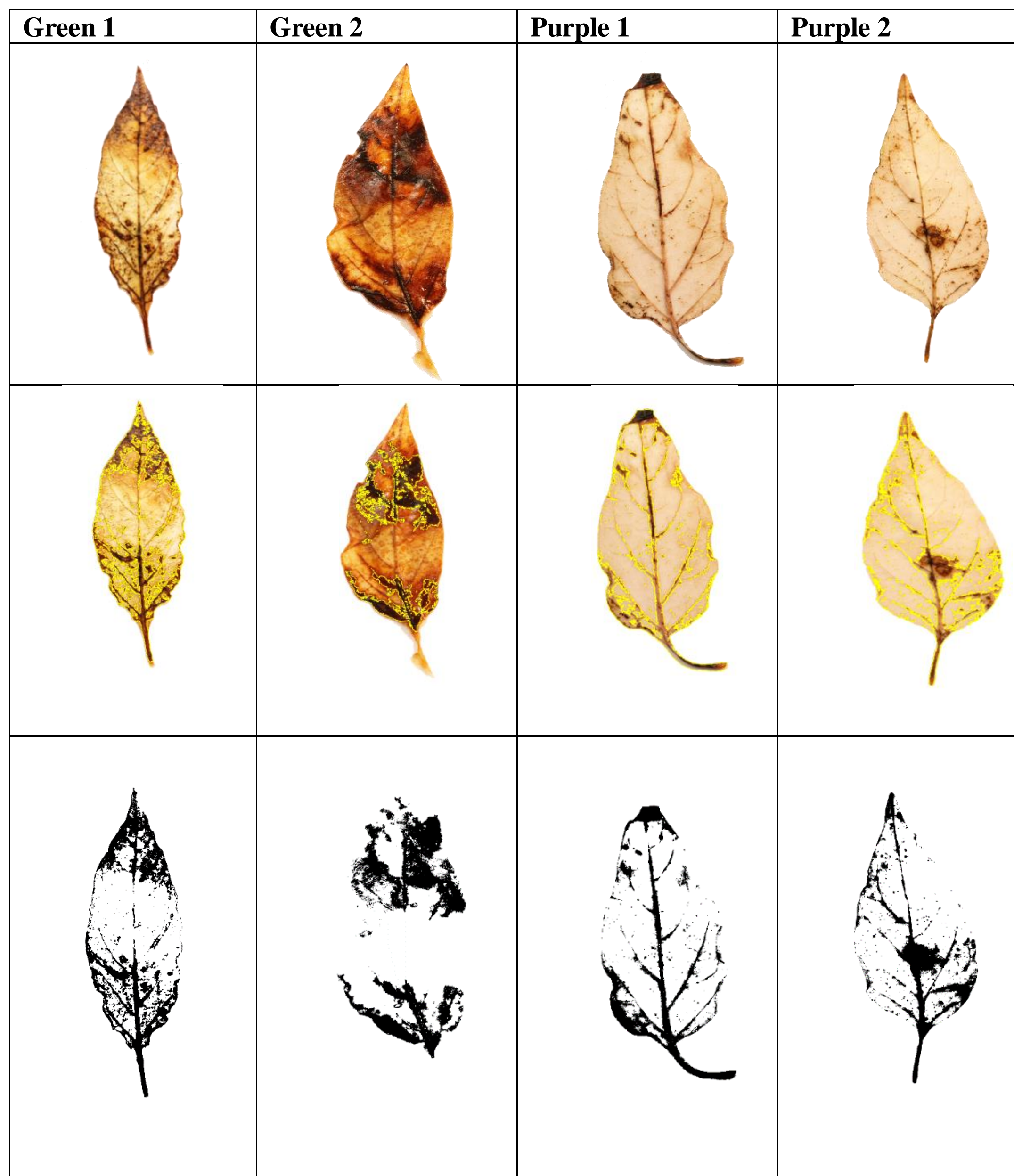
This explains that the anthocyanins in purple leaf acted against the infestation and reduced it infestation to spread so that we get low staining percentage in purple leaves compared to the

green leaves. Based on this article (Venisse et al., 2001) NBT assay was performed which explains during pathogenicity ROS increases NBT value increase. Whereas as in purple leaves anthocyanins act against ROS reduce the NBT value.

4.3.2. DAB (3,3′-diaminobenzidine)

DAB staining is to visualize ROS accumulation as ROS are the key indicators of oxidative stress and plant defense responses. DAB detects hydrogen peroxide (H_2O_2) which is a later ROS accumulation and turns the infected leaf to brown or reddish brown due to polymer deposit (fig 20).

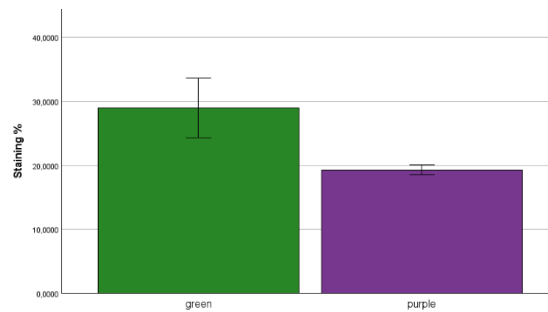
Figure 20. DAB staining in green and purple leaves which are infected with *Fusarium*



green 1 and green 2 showing the intense brown deposition (fig 20). High hydrogen peroxide accumulation low antioxidant defece. Where as in purple 1 and purple 2 there is less brown stain (fig 20) compared to the green leaves is due to the lower ROS accumulation because anthocyanins are ROS scavengers.

Figure 21. DAB Staining percentage (1) and graphical (2) representation of green and purple leaves.

Staining %	Leaf color
33,64887	Green
24,29985	Green2
22,60983	Green3
18,5322	Purple
20,05346	Purple2
17,43695	Purple 3



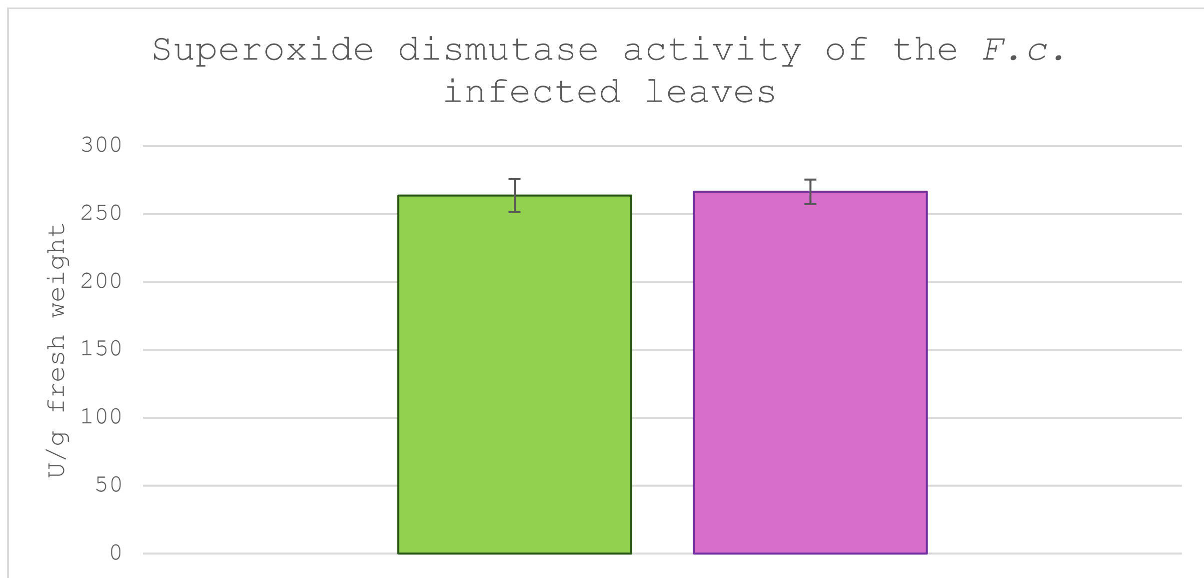
1 **2**

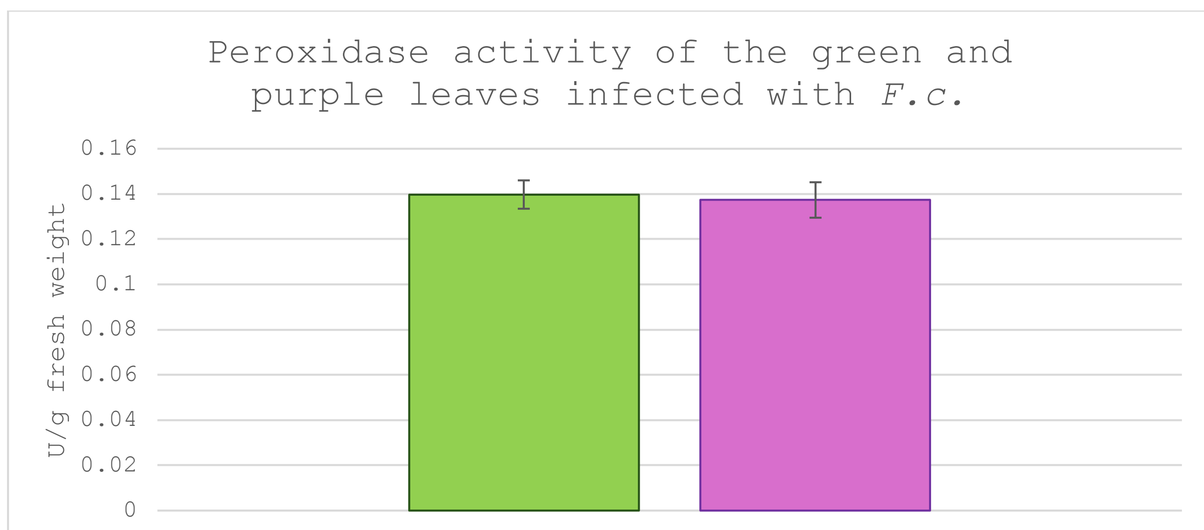
Purple leaves showing 17-20 DAB staining area which means lowers H_2O_2 accumulation explains strong antioxidant protection by anthocyanins. Green leaves show 24-33% DAB-stained area means higher H_2O_2 buildup, greater oxidative stress and weaker ROS scavenging (fig 21/1). Also, we observe the same differences from the graph (fig 21/2).

This explains that the anthocyanins in purple leaves acted against the infestation by reducing hydrogen peroxide and prevented infestation to spread so that we get low staining percentage in purple leaves compared to the green leaves. Based on this article (Daudi et al., 2012) DAB staining was performed which explains, during pathogenicity DAB value increases with increase in hydrogen peroxide. Whereas as in purple flowers anthocyanins act against hydrogen peroxide reduces the DAB value.

In *Fusarium culmorum*–infected leaves, no significant differences were observed between the green and purple leaves of the same genotype in either superoxide dismutase (SOD) or peroxidase (POD) activity (fig 22). This indicates that both leaf types activated their enzymatic antioxidant defense systems to a similar extent in response to the infection. The comparable SOD and POD levels suggest that the oxidative stress generated by *F. culmorum* was effectively neutralized in both tissues, and the presence of anthocyanins in the purple leaves did not markedly alter the overall enzymatic response. These results imply that under *Fusarium* induced stress, both genotypes relied on a comparable antioxidant strategy to mitigate reactive oxygen species.

Figure 22. superoxide dismutase (SOD) and peroxidase (POD) in green and purple leaves from same genotype.





5. CONCLUSION

This study explains that anthocyanin biosynthesis in *Capsicum annuum* is strongly influenced by the light exposure and epigenetic regulation through DNA methylation. The flowers which are uncovered exhibited higher Total Polyphenolic Content (TPC). Ferric Reducing Antioxidant Power (FRAP) and Total Flavonoid Content (TFC) values indicating enhanced pigment accumulation and antioxidant activity under illumination. This increase correlates with the activation of anthocyanin biosynthesis genes through the light responsive MYB-BHLH-WD40 (MBW) transcriptional complex. Conversely, covered flowers showed reduced TPC, FRAP and TFC evidence low anthocyanin accumulation, likely due to lower stimulation of anthocyanin biosynthesis gene and absence of light. The methylation effects the anthocyanin biosynthesis at CCGG sites as revealed by Methylation Sensitive Amplified Polymorphism (MSAP) analysis. The results confirmed that hypermethylation represses key genes in the anthocyanin biosynthesis pathway, leading to decline pigment accumulation. Methylation is observed more in covered flowers than in the uncovered flowers which explains, light oppose the methylation.

Additionally, detached leaf assays demonstrated that anthocyanin-rich (purple) tissues exhibited greater tolerance against *Fusarium culmorum* infection, confirming the protective antioxidant role of anthocyanins.

Overall, this research establishes a clear connection between light induced demethylation, transcriptional activation of structural and regulatory genes and anthocyanin accumulation in purple peppers, emphasizing anthocyanins photoprotective and antifungal significance.

6. SUMMARY

This thesis work investigates on how light impacts the anthocyanin accumulation in purple peppers (*Capsicum annuum)*, function of the structural genes (EBGs and LBGs) and regulatory genes (MBW complex) in anthocyanin biosynthesis. To confirm that light regulates the anthocyanin accumulation some analytical measurements and DNA methylation analysis was conducted. Also, studied how anthocyanins acted against the *Fusarium culmorum* and antioxidant potential towards ROS during infestation.

In the research involved covered and uncovered flowers to examine how different light conditions results in anthocyanin biosynthesis. From the analytical tests- Total Polyphenolic Content (TPC), Ferric Reducing Antioxidant Power (FRAP) and Total Flavonoid Content (TFC) gave high values in flowers which are exposed to the sun compared to the light eliminated flowers quantified the changes in phenolic compounds and antioxidant levels. By Methylation Sensitive Amplified Polymorphism (MSAP) assessed epigenetic modification, in the results showed that light exposure reduces DNA methylation particularly at CCGG sites. Detached leaf assays inoculated with *Fusarium culmorum* tested the antifungal defense in anthocyanin-rich tissues.

From all the results the findings establish that light not only enhances anthocyanin biosynthesis by demethylation DNA and activating transcriptional regulators but also strengthens the plant's antioxidant and pathogen resistance mechanism. Purple leaves with higher anthocyanin content displayed greater oxidative balance and lower fungal infection rates than green leaves. This study also emphasizes the importance of anthocyanins in plants and nutritional supply to the humans.

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List of figures

Figure 1. plant development in *C. chacoense* **A.** monopodial vegetative growth **B.** 1st dicotomy of the main stem and start of sympodial growth **C.** initial branching with three branches……………………6

Figure 2. flower morphology in *Capsicum* species **A** *C. rabenii* ***B*** *C. annuum var. glabriusculum* **C** *C. lanceolatum* **D** *C. schottianum* **E** *C. frutescens* **F** *C. eximium* **G** *C. eshbaughii* **H** *C. cornutum* ***I*** **C**. *galapagoense* **J** *C. recurvatum* **K** *C. cardenasii* **L** *C. lycianthoides* **M** *C. chacoense* **N** *C. baccatum var. baccatum* **O** *C. caballero* ………………………………………………………………………………7

Figure 3. fruit color diversity in A) brijal, pepper and tomato B) variation in fruit color in tomato………………………………………………………………………………….8

Figure 4. Transcriptional regulatory network of anthocyanin biosynthesis in plants………………….9

Figure 5. Representative fruit color of pepper. W, White; I, Ivory; Y, Yellow; LG, Light Green; DG, Dark Green; O, Orange; R, Red; P, Purple; PB, Purple Black……………………………………...…11

Figure 6. Simplified scheme of flavonoid pathway, including phenylpropanoid pathway, the anthocyanin branch and other subgroups of flavonoid end-products…………………………………14

Figure 7. General chemical structure of anthocyanidins and the six most common anthocyanidins in *Solanaceous* vegetables, indicated by "X" …………………………………………………...15

Figure 8. schematic representation of environmental factors regulating anthocyanin biosynthesis through MBW complex……………………………………………………………………………….17

Figure 9. In all 1 to 5 plants two flowers are covered with silver foil to elimenate the illumination from five plants and remaining flowers are left uncovered…………………….20

Figure 10. inoculation of *Fusarium culmorum* on green leaves and observed for 4 days…..27

Figure 11. inoculation of *Fusarium culmorum* on leaves purple and observed for 4 days….28

Figure 12. TPC of week 0, covered/uncovered week 1 and week 2 plants………………….30

Figure 13. The FRAP value in week 0, week 1 and week 2 covered and uncovered flowers.31

Figure 14. TFC of the week 0, week 1 and week 2 covered and uncovered flowers………..32

Figure 15. methylation band patterns…………………………………………………….….33

Figure 16. In the above gel images (1 and 2) we observed the function Of HpaI and MspII in pepper flowers who are in light and dark phases. L and D represent light and dark…………………..34

Figure 17. NBT stained green leaves which are infected with *Fusarium*…………………...35

Figure 18. NBT stained purple leaves which are infected with *Fusarium*……………….….36

Figure 19. NBT staining percentage (1) and graphical (2) representation in green and purple leaves………………………………………………………………………………...….36

Figure 20. DAB staining in green and purple leaves which are infected with *Fusarium*…..37

Figure 21. DAB Staining percentage (1) and graphical (2) representation of green and purple leaves……………………………………………………………………………………38

Figure 22. superoxide dismutase (SOD) and peroxidase (POD) in green and purple leaves from same genotype……………………………………………………………………….39

List of tables

Table 1: Sequences of adapters, pre-selective, and selective primers……………………….26

Table 2. Methylation patterns in light and dark phases……………………………...…34

MATE Organizational and Operational Regulations
III. Requirements for Students
III.1. Study and Examination Regulations
Appendix 6.13: The MATE Uniform Thesis /thesis / final thesis / portfolio guidelines
Annex 4.2: Declaration of public access and authenticity of the thesis/thesis/dissertation/portfolio

DECLARATION

the public access and authenticity of the thesis/dissertation/portfolio[1]

Student's name: KOSANAM JERUSHA.
Student's Neptun code: A2DKPF
Title of thesis: The effect of Light on anthocyanin accumulation, antioxidant Capacity and DNA methylation in capsicum annuum.
Year of publication: 2025
Name of the consultant's institute: Institute of genetics and biotechnology
Name of consultant's deparment: Institute of genetics and biotechnology.

I declare that the final thesis/thesis/dissertation/portfolio[2] submitted by me is an individual, original work of my own intellectual creation. I have clearly indicated the parts of my thesis or dissertation which I have taken from other authors' work and have included them in the bibliography. Furthermore, I declare that the artificial intelligence tools (e.g. text generation, linguistic correction, translation, data analysis) used during the preparation of the thesis did not substitute my own research and creative work; their use was indicated either in the list of sources or in the methodology section, and I acted in accordance with professional and ethical expectations.

If the above statement is untrue, I understand that I will be disqualified from the final examination by the final examination board and that I will have to take the final examination after writing a new thesis.

I do not allow editing of the submitted thesis, but I allow the viewing and printing, which is a PDF document.

I acknowledge that the use and exploitation of my thesis as an intellectual work is governed by the intellectual property management regulations of the Hungarian University of Agricultural and Life Sciences.

I acknowledge that the electronic version of my thesis will be uploaded to the library repository of the Hungarian University of Agricultural and Life Sciences. I acknowledge that the defended and

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will be available publicly and can be searched in the repository system of the University.

Date: 03 year 2025 month NOV day

K. Jerusha.
Student's signature

[1] While keeping the appropriate thesis type, all other types are to be removed.
[2] While keeping the appropriate thesis type, all other types are to be removed.0

DECLARATION

KOSANAM JERUSHA (name) (student Neptun code: A2DKPF)
as a consultant, I declare that I have reviewed the final thesis/thesis/dissertation/portfolio[1] and that I have informed the student of the requirements, legal and ethical rules for the correct handling of literary sources.

I recommend / do not recommend[2] the final thesis / dissertation / portfolio to be defended in the final examination.

The thesis contains a state or official secret: yes no*[3]

Date: 03 year 2025 month NOV day

[signature]
insider consultant

---

[1] The other types should be deleted while retaining the corresponding thesis type.
[2] The appropriate one should be underlined.
[3] The appropriate one should be underlined.

Declaration of Students and Doctoral Candidates on the Use of Artificial Intelligence (AI)"

1. general information:

Name of the student:	KOSANAM JERUSHA.
Neptun ID:	A2DKPF
Level of program (mark with X):	☒ BSc/BA ☐ MSc/MA ☐ Doctoral School (PhD) ☐ Other: ....
Name and code of the subject*:	Degree thesis
Title of the work:	The effect of Light on anthocyanin accumulation, antioxidant capacity, and DNA methylation in Capsicum annuum.

* Not required to be completed in the case of a doctoral dissertation.

2. Declaration on the Use of AI

I, the undersigned, fully aware of my ethical responsibility, make the following declaration:

(Please choose one of the options below!)

☐ A) I have not used any artificial intelligence system or service.

(If you selected this option, completing the subsequent tables is not required.)

☐ B) I have used an artificial intelligence system or service.

(Please fill in the relevant tables!)

3. Details of Artificial Intelligence Usage

TABLE I: Assistant or Minor Usage (e.g., translation, language proofreading, brainstorming, etc.)

(For these uses, attaching the specific prompts and responses is not required.)

Purpose of Use	Name and Version of the AI Tool Used	Affected Section (if not applicable to the entire text)

TABLE II: Significant Content Contribution (e.g., generating an entire figure or a longer text section)

(In these cases, documenting the key prompts used and the raw responses provided by the AI, and attaching them as an appendix to the work, is required.)

Purpose of Use	Name, Version, and Access Information of the AI Tool Used	Exact Number of the Affected Chapter / Figure / Table	Entry Number of the Appendix Containing the Prompt Log

3/A. Additional Rules Prescribed by the Lecturer (if any)

If the instructor or supervisor of the course has established specific rules or expectations regarding the use of AI tools, please summarize them in the field below:

For example: prohibition of AI use for certain types of tasks; only specific tools are permitted; different citation requirements; documentation format, etc.

Rules Prescribed by the Lecturer or Supervisor

As a thesis adviser I have no tools provided by the university to check the amount of AI usage.

4. Declaration Applicable to All Students:

I declare that I have critically reviewed, edited, and incorporated any content potentially generated by AI in all cases. I take full responsibility for every element of the submitted work, including its originality and scientific validity. I acknowledge that the Hungarian University of Agriculture and Life Sciences may check the submitted work with an artificial intelligence detector and may initiate proceedings if my declaration is found to be false or incomplete.

Place and Date: Gödöllő, 2025. NOV month 3 day

K. Jerusha — **Signature of the Student**

Kovács Zsófi — **Signature of the Advisor/Supervisor**