THESIS

DALLI SRUTHI LAYA

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Hungarian University of Agriculture and Life Sciences

Szent István Campus

Institute of Genetics and Biotechnology

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The Effect of Lightning on Anthocyanin Biosynthesis of *Capsicum Annuum*

Insider consultant: Zsófia Kovács,
Senior Lecturer

Insider consultant’s
Institute/department: Institute of genetics and Biotechnology, department of Genetics and Genomics

Created By: DALLI SRUTHI LAYA
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1. INTRODUCTION AND OBJECTIVES

Capsicum annuum L., commonly known as pepper, is an important vegetable and a spice crop with global importance. It belongs to the *Capsicum* genus, which consists of more than 30 species, *C. annuum* is one of the five domesticated species cultivated primarily for consumption purposes. *Capsicum annuum* has been used since approximately 7500 BC in the northern hemisphere, making it one of the first known farmed crops in human history. In addition to its culinary importance, *Capsicum annuum* is well known for its therapeutic characteristics, which include anti-inflammatory, analgesic, antioxidant, hypoglycaemic, antifungal, and antibacterial properties.

Nowadays, there is an increasing trend towards novelties and functional foods, hence purple is a frequently observed colour in chili peppers. The fascinating purple or blue hue of pepper results from the aggregation of diverse amounts of anthocyanins, primarily delphinidin molecules. In addition to playing important roles in plant and microbial signalling, anthocyanins are also antimicrobials, feeding deterrents, and the cause of male fertility in some species. They are crucial for biological defence, photoprotection, and governing the relationships between plant cells and microorganisms. Further, it has been observed in the case of purple tomato studies that anthocyanins often act as the first line defence against the pathogen attacks. When the accumulation of anthocyanin had been reduced in tomatoes, the areas with less anthocyanin were more susceptible to *Botrytis cinerea* than the purple areas. One of the most substantial environmental elements impacting the accumulation of anthocyanins is light. Many plant species produce more anthocyanins when exposed to high light levels. This elevated level of anthocyanin can lead to more antioxidant capacity, more polyphenolic content, therefore might result in enhanced stress tolerance as well as better nutritional profile. With the development and increasing availability of genetic and genomic tools, resulting in expanding our understanding of *C. annuum*, making it more possible to initiate breeding programs which aims at improving resistance to biotic and abiotic stresses and also improving the fruit quality traits therefore, *Capsicum annuum* continues to be in the main focus of point for scientific research, agronomic development, and nutritional innovation.

Therefore, our objective is to study:

- the total polyphenolic content,
- total flavonoid content,

- antioxidant capacity of leaves - that are either illuminated or covered – of the same genotype, to test the effect of light on this biosynthetic pathway.

Further, methylation pattern differences are also being monitored in the covered, illuminated leaves.

Lastly, to test the efficacy of purple or green leaves of the same genotype against pathogen infections, a detached leaf assay is carried out, to rule out if anthocyanins are adding to the overall stress tolerance against *Botrytis cinerea.*

1. LITERATURE REVIEW

2.1 Solanaceae

Solanaceae family is one of the largest and most economically important families which belong to angiosperms, which includes important food, spice and medicinal drug plants (Ganaie et al., 2018). This family representing around 90 genera of flowering plants which consists of more than 3500 species globally making it cosmopolitan making its distribution mainly represented in tropical and temperate regions (Albuquerque et al., 2006). The most diversity of this species occurs in south America where this area is also recognized as the origin of this family (Albuquerque et al., 2006).

Solanaceae also called as a cluster of nightshade flowers, the nightshade, family of flowering plants. The name nightshade is given to solanaceae family due to the presence of the poisonous alkaloids in some species of the family (Encyclopaedia Britannica, 2025).

2.1.1 Physical description

Plant members of Solanaceae are typically trees, shrubs and herbs, although there are some climbers, vines and epiphytes. Leaves of the family are usually simple, sometimes they can be greatly lobes, petiolate or alternate to opposed. They have herbaceous, leathery or modified spines type of leaves. Cymose and axillary are the most prevalent inflorescences, whereas they can be reduced to a single flower. Flowers are typically 5 membered (pentamerous), bisexual, medium sized, single or clustered, regular or irregular and they have cymose or axillary inflorescences. Majority of Solanaceae species are hermaphrodites or bisexual, but some are monoecious or dioecious, with a combined calyx and perianth (Zandi & Ix, 2014). The family also includes a five-lobed calyx and a sympetalous corolla, both of which have five lobes that are either induplicate-valvate or plicate in the bud. Except for a few taxa, epipetalous stamens are perfect, equal in length, and placed alternately with petals. They are often as many as petals, though occasionally less. Rarely, three or five carpels can merge to form the gynoecium, which is made up of two joined carpels. One characteristic that sets Solanaceae apart from Convolvulaceae is the abundance of ovules and seeds. The majority of solanaceous members have a basic set of twelve chromosomes (Ganaie et al., 2018). Type of fruits are berries, capsules or drupes. Seeds are predominantly small round and flat (Zandi & Ix, 2014).

2.1.2 Classification and Importance

The family Solanaceae is referred to as "the night shade family" since it is derived from the solanum gene. One of the biggest and most significant families of angiosperms in terms of economics is the Solanaceae, which includes significant food, spice, and medicinal plants. It belongs to the order of solanales in the Astrid group of dicotyledons [magnolipsida]. It has over 2700 species, 98 genera, and a wide variety of habitats, morphologies, and cologies. With the exception of Antarctica, the family is found across the planet. South America and Central America have the most variety. Although shrubs are abundant and trees are rare, the majority of members are upright or ascending annular and perennial herbs (Shelar et al., 2023)

ECONOMIC IMPORTANCE

The Solanaceae is one of the most crucial plant families that has extreme economic importance. Solanaceae is one of the main foods providing plant families. Fifteen genera yield species that are consumed globally. Among them, only four genera encompass economically important cultivated food crop species. The majority belong to the genus Solanum, while Capsicum, Physalis, and Lycium account for the remaining cultivated crop species. These genera, among others, include species that are semi-cultivated, regarded as beneficial weeds, or foraged from the wild (Samuels, 2015).

This family plays a main role in being the source of diverse food plants. The majority of food species around 150 plants of solanoideae belongs to the four genera as such: *Solanum, Capsicum*, *Jaltomata* and *Physalis.* Cultivation types include commercial cultivation, subsistence farming, semi cultivation, tolerated weeds and wild harvesting (Samuels, 2015).

GENUS: SOLANUM (largest contribution to food crops**)**

- *Solanum tuberosum* (Irish potato) – Globally important staple crop
- *Solanum lycopersicum* (tomato) – Widely cultivated and has high economic value
- *Solanum melongena* (eggplant) – common vegetable in Asia, Africa and Mediterranean

GENUS: *CAPSICUM*

- *Capsicum annum* (bell pepper, chili pepper)
- *C. chinese* (Habanero, Scotch bonnet)
- *C. frutescens* (Bird pepper, Tabasco pepper)
- *C. baccatum* (aji)
- *C. pubescens* (rocoto)

GENUS: JALTOMATA (all of them are recorded edible)

- *J. procumbens* (uva de monte)

GENUS: PHYSALIS

- *P. peruviana* (uchuva)
- *P. philadelphica* (tomatillo) (Samuels, 2015)

2.2. *Capsicum annuum*

The botanical term *capsicum* was proposed by Fuchs in 1543, but it was later adopted in 1753 by Linneo. The name would be the neolithic derivation of the Greek word called "Capsa" which implies the peculiar shape of the fruit. This crop was introduced to Europe by the discoverer of America, Christopher Columbus in the fifteenth century and later it was spread to Africa and Asia (Tripodi & Kumar, 2019).

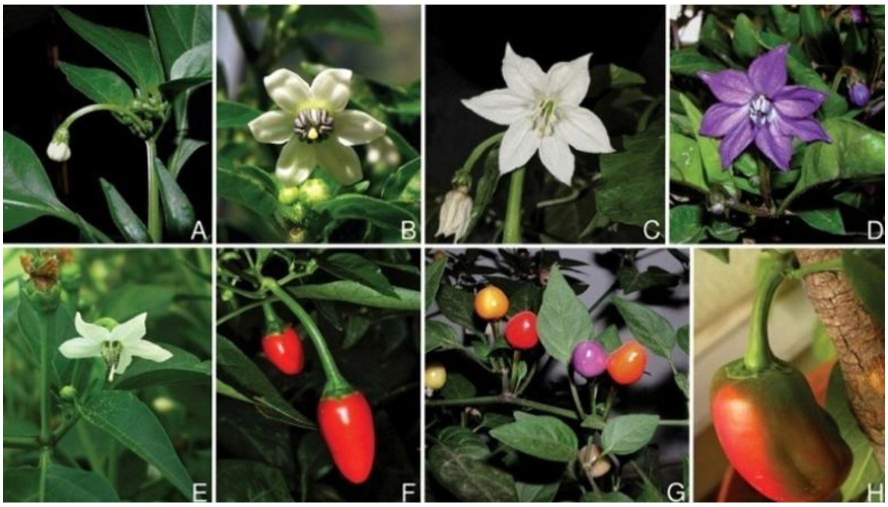
Capsicum species are diploids, with most of them having 24 chromosomes, however several wild species showed 26 chromosomes.(Directorate, 2006; Swamy, 2023). Cytological studies of *Capsicum annuum* showed that these species showed the mitotic chromosome number as 2n=24 at the early metaphase (Wahua et al., 2013).

The genus *Capsicum*, under the Solanaceae family, is characterized by its shrubby growth form, actinomorphic flowers, unique truncate calyx regardless of its appendages, anthers that dehisce via longitudinal slits, nectarines located at the base of the ovary, and its diverse, typically pungent fruits. *Capsicum annuum var. glabriusculum* is considered as the origin of all the chilli species in the world. The wild ancestor of *C. annuum* is believed to be the bird pepper, perhaps domesticated in southern Mexico and transported to Europe in the fourteenth century (Swamy, 2023).

2.2.1 Botanical description

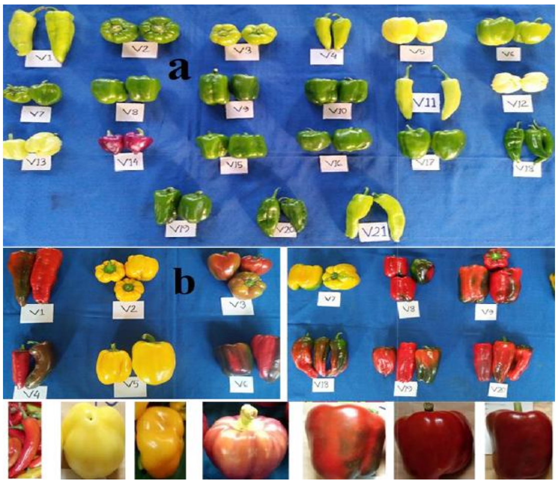
Capsicum annuum is generally grown as herbaceous annual plant in the temperate regions. Nevertheless, in tropical areas, it is considered ecologically a perennial shrub which can live from a few years to a few decades. Also, it can be grown as a perennial in a climate-controlled areas like greenhouses (Directorate, 2006). The name annuum which means annual, the plant can survive several seasons in the absence of winter frost and can grow into a large perennial shrub. These species can withstand most climates but are found most productive in warm and dry climates (Swamy, 2023).

Figure 1: *Capsicum annuum var. annuum*. A. flower bud on pendent pedicel; B. flower with connivant anthers; C. flower with hexamerous corolla (note nectar droplets on the limb) and style near the same length as the anthers; D. flower with heptamerous purple corolla; E. flower with pentamerous corolla and style exceeding the anthers; F. mature fruits on pendent pedicels; G. mature fruits on upright pedicels); H. mature fruits on pendent pedicels (Swamy, 2023).



Capsicum annuum, this species is one of the most distributed species in the world. The fruits of this plant show a considerable diversity, and their sizes ranges from 1 to 25 cm (Naegele et al., 2016) and their spice level ranges from very mild, slightly sweet bell peppers to small but very hot jalapeño. The form, sizes and colour of the fruits have various aspects and sides as shown in fig 1. and 2., the branches are densely branched and can grow up to 60 cm or 24 inches tall (Swamy, 2023).

Figure 2. - Variation of fruit colour, shape and size at(a) intermediate stage and (b) mature stage (Swamy, 2023).



The sizes of the fruit can be enormously thick walled to small berry like pods. *Capsicum* displays a wide range of fruit and plant colours(García-González & Silvar, 2020). The fruits which are berries can be of various colour, but the most dominating ones are the green, yellow and they turn orange or red when ripe (fig. 2.). The ripening fruits are usually red, but some varieties may be ripe to yellow, orange, peach, brown or in same rare cases purple. Single flowers of this plant are off-white colours however sometimes purplish colour (Andrade et al., 2020). The fish pepper plant has a white and green variegated leaves and they have striped peppers, while the Pimenta da Neyde pepper is known for its black foliage and dark purple peppers (Swamy, 2023).

2.2.2 Reproductive Biology

Reproductive organs:

FLOWER: *Capsicum annuum* produces a white large flower with calyx teeth starting at the axil of the branching node and subsequent flowers forms at every node. The flowers are solitary, auxiliary, bisexual, hypogynous, actinomorphic, pedicellate and usually pentamerous. The flowers are complete as there is calyx which are 5 in number, corolla consists of 5 bell shapes petals and presence of both male and female sex organs (fig. 1.) (Directorate, 2006; Yadav et al., 2022). The radius of the flower is around 4.5mm to 7.5mm (Directorate, 2006). Androecium has introrse anthers, gynoecium has 2 carpels, syncarpous with obliquely placed placenta. It has a superior ovary with 2 or 4 celled with number of ovules in each locule with an axile placentation (Yadav et al., 2022).

FRUIT

The time from anthesis to a full-grown fruit is different considerably from different pod types. Cultivated fruit reaches the mature green stage in around 35 to 50 days after the pollination. Fruit maturity depends on the type of cultivar and environmental conditions before and during maturation (Directorate, 2006).

When it comes to the fruit there is extreme diversity in its shape, size, wall thickness, and fleshiness, colour and the pungency which is determined by genetic (P. Li et al., 2022) and environmental factors. Within this number of varieties, the diversification of the shape of the pods stands out. For e.g. bell shaped, globose, sausage shaped, ovoid, conical, cylindrical, smooth, grooved, lumpy, or wrinkled. The length of the pod varies from 1 cm to 32.5 cm. the length of the pedicel also varies greatly in different pod types, and therefore the fruit can be erect or pendant. Colour of the fruits range from green, yellow, orange and red to purple, brown, black and white as well (Directorate, 2006).

WHAT IS RESPOSIBLE FOR THE DIFFERENT COLOURS IN *CAPSICUM*?

Carotenoid pigments which are formed during the ripening of the fruit results in yellow, orange and red colours. More than 30 types of carotenoids are identified in *capsicum* fruits. Some of the carotenoids are provitamin A which is essential for human nutrition, and the oxygenated carotenoids that have been recorded as anti- cancer agents (Tepić & Vujičić, 2004).

Current research shows that there are three kinds of pigments that are responsible for the colour of the plants namely anthocyanins, carotenoids and betaines (Lu & Gong, 2018). Anthocyanins are pigments that are spread widely in nature which are responsible for the colours like purple, blue, red and orange in fruit, stem, leaf, flower and tuber of many species(Patra et al., 2022). Studies on peppers which has anthocyanins are very rare, but a few papers have described the presence of anthocyanins in pepper fruits. For example, in an Israeli cultivar, delphinidin was reported as the main anthocyanin (Borovsky et al., 2004). Similarly, delphinidin-3-trans-coumaroylrutinoside-5-glucoside (nasunin) and delphinidin-3-cis coumaroylrutinoside-5-glucoside were identified as the primary anthocyanins in a German chili pepper (*C. annuum*), among others (Sadilova et al., 2006). Recently, it was discovered that the only anthocyanin found in violet and black chili pepper fruits is delphinidin-3-p-coumaroylrutinoside-5-glucoside (Aza-González & Ochoa-Alejo, 2012). (Kovács et al., 2022) study showed that anthocyanins are present in pepper fruits and add to antioxidant capacity, with levels differing according on genotype and ripening stage.

Fruit growth and development

Capsicum annuum, which is cultivated widely in many temperate, subtropical and tropical regions in the world. While in its growth period many climatic and soil factors can affect the growth development of peppers like air temperature, light intensity, precipitation and soil conditions. Temperature is one of the major environmental factors that affect the development of peppers in processes like flowering, fruit set and fruit growth. It was found that the temperature range of 20-25°C is favourable for the vegetative growth, fruit development and fruit quality (Oh & Koh, 2019). Light also plays a major role in plant development of peppers such as stem elongation, leaf expansion, branching patterns and also in reproductive processes like flowering and fruit development (Wen-Feng Nie 1, 2023).

Capsicum fruits possess metabolites like capsaiconoids, and carotenoids produced over the process of maturation. This metabolic production can cause changes in flavour, colour, texture and aroma of the fruits to make them more attractive (Fernando G. Razo-Mendivil, 2021).

Changes during Maturation and ripening

Physiological changes in pepper:

After 40- 45 days of anthesis, it was found that the fruit diameter, length and weight were increasing steadily and after that the growth slowed. At 65 days after anthesis (DAA), the fruit weighed 45.52 and at 75 days after anthesis the fruit weighed 45.53 indicating the growth stabilization. Dry matter content of the seeds increased along with the age of the fruit reaching its maximum at 75 days after anthesis, when the fruits were completely red. There is a seed dehydration during the seed maturation, as seed water content decreased from 91.8% at 20 DAA to 47.3% at 75 DAA (De Souza Vidigal et al., 2010).

Similar trends were observed in new Mexican pepper's fruit growth which has sigmoidal pattern with a fast rise up to 33–40 days after flowering and stability at the mature-green stage around 60 DAF. Indicating a non-climacteric ripening pattern, between 60 and 70 DAF, respiration slowly dropped as fruits became red, whereas chlorophyll decreased, firmness declined, ethylene production increased, and β-galactosidase activity increased. These alterations reflect peppers' natural ripening and maturation processes and are consistent with the growth stabilization and seed dehydration seen at 65–75 DAA (Biles et al., 2019).

The sweet pepper variety "BARI Misti Morich-1" showed a similar developmental pattern with fruit growth and quality characteristics until after 45 days of anthesis, at which point growth decreased and the fruit attained its optimal physiological maturity (Rahman et al., 2014).

Biochemical changes:

Accumulation of dry matter in the seeds peaked at 75 DAA, suggesting the end of seed filling period, at this period of time, protective molecules like LEA proteins and soluble sugars like sucrose, raffinose, stachyose accumulated (De Souza Vidigal et al., 2010). Ascorbic acid i.e., vitamin C content decreased from 68 mg/100 g to 56 mg/100 g during the harvest window. At 45 DAA, the vitamin C content is 58 mg/100 g (Rahman et al., 2014).

Early in fruit development (10-30 days post anthesis), anthocyanins, especially delphinidin, accumulated in the pericarp of coloured cultivars, peaking before progressively decreasing as the fruit grew. During this phase, genes involved in anthocyanin production and vacuolar sequestration, including as F3'5'H, DFR, UFGT, and GST, were significantly expressed. This shows that anthocyanins function as early-stage protective pigments, perhaps providing antioxidant defence and UV protection during the early stages of fruit development (Aza-González et al., 2013).

Pepper fruit turns red as it ages because red ketocarotenoids like capsanthin and capsorubin are newly generated while chlorophylls and chloroplast carotenoids like lutein and neoxanthin break down. This change signifies a crucial stage in ripening: the transformation of chloroplasts into chromoplasts. Some cultivars have brownish tints because of overlapping red and green pigments caused by inadequate chlorophyll degradation (Hornero-Méndez et al., 2000).

Harvesting

When studies about harvest timings, nine harvest timings were considering. The harvests were done with days after transplanting. The highest fruits around 95.34 fruits were found at 163.21 days after transplanting. It was concluded that the harvest should be carried out within a 15-day interval, if it was longer than that interval the fruit rot occurs (Rahman et al., 2014).

2.2.3 Post Harvest

Post harvest technology for extending shelf life has gained importance significantly in recent years with increasing demand for fresh fruits and vegetables (Bosland et al., 2005). It is said that optimum post-harvest storage condition can improve the post-harvest quality and shelf life of horticultural crops (M.Anusha et al., 2024) ; (Ziv & Fallik, 2021). There were several technologies installed on post-harvest peppers like forced air-cooling (FAC) and modified atmosphere packaging (MAP) showed effects on shelf life, physiochemical quality, health promoting properties of bell pepper. However, regardless of treatments, some varieties of peppers showed significant weight loss, effect on firmness of the fruit during cold storage (Yeboah et al., 2023).

Identification of Rot Fungi on Post-Harvest of Pepper:

Fungi have been one of the most important spoilage organisms found on pepper fruits. There are characteristic fungi rots, such as *Penicillium, Aspergillus* and *Alternaria* and some zygomycetes like *Mucor* and *Rhizopus*. A study in Nigeria shows that multiple fungi, mainly *Aspergillus*, *Fusarium*, *Colletotrichum*, and *Bipolaris*, are responsible for rot in peppers (Akinyemi & Liamngee, 2018). These species produce many spores, and they can take advantage of any damage or bruising and can attack the fruit from harvest to consumption stages. Research showed that fungi can make its way into the host tissue through natural openings as lenticels, stomata and even through unbroken epidermis (Akinjide et al., 2017).

Aspergillus niger and *Rhizopus stolonifera* were the primary causes of spoilage. It can be caused due to the poor handling of post-harvest peppers and storage conditions like environment factors such as humidity and temperature. High moisture, moderate temperature and acidic environments are favourable for the fungi growth. Unsterilised containers allowed easy contamination. By these studies, it was advised that post-harvest safety and conditions are absolute necessity to avoid any kind of spoilage and damage (Akinjide et al., 2017).

The study of (Frimpong et al., 2019) shows that post-harvest pepper fruits are extremely vulnerable to fungal infection, with Aspergillus niger and *Rhizopus stolonifera* being the most prevalent and numerous rot-causing fungus. Fungal spores may easily infect peppers during harvesting, transit, and storage, especially in environments with high humidity and moderate temperatures (Frimpong et al., 2019). For example *Botrytis cinerea* grows rapidly under favourable environmental conditions such as 18-24 °C temperature and relative humidity > 93% and is most likely to infect and spread on pepper fruits (Yang et al., 2023).

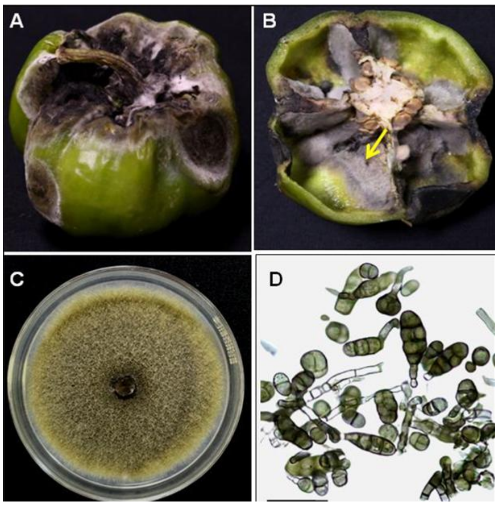
Soft rot of postharvest pepper

Post harvest soft rot, which is caused by *Pectobacterium carotovorum*, is one of the most important bacterial diseases during storage and transportation. These bacteria attack pepper when they are most susceptible to microbial infection, which is mainly after the harvest during storage especially when the fruits are injured by insect feeding and, when peppers are exposed to warm and humid conditions etc, which can cause severe economic losses (Li et al., 2024).

Postharvest fruit rot

Postharvest fruit rot in bell pepper is often caused by the fungus *Alternaria alternata* (fig. 3.). This infection does not show until the fruit ripes however it effects when the fruit is green and unripe, because it stays dormant during this period. This fungus enters mainly through wounds or natural openings and can survive in warm and humid conditions.it becomes active after the fruit ripe and leading to fruit rot (Balamurugan & Kumar, 2023).

Figure 3. - Fruit rot symptoms, cultural and conidial morphology of A. alternata isolated from bell pepper: Fruit rot infected green bell pepper (A); Cross- sectioned fruit showing internal fruit and seed rot (B, yellow arrowhead); Greyish to olive green fungal growth on PDA (C); Dark brown, obclavate/spheroidal shape conidia with 1-3 longitudinal and 3-5 transverse septa (D); Scale Bar = 20 µm (Balamurugan & Kumar, 2023).



Delayed fruit ripening in purple pepper fruit

A study showed that when the application of blue light is increased it also increased the anthocyanin levels and therefore delayed the fruit ripening (Zhou et al., 2024). Fruit ripening is delated under the blue light, as seen by slower sugar accumulation, retained chlorophyll and a lower expression of ripening related genes. It is believed that the ethylene production is repressed under blue light and therefore leading to delay the ripening. However, the anthocyanin levels decreases as the fruit starts to ripe, this technique of blue light is valuable in post-harvest storage application but not for producing ripe anthocyanin rich fruits (Liu et al., 2022).

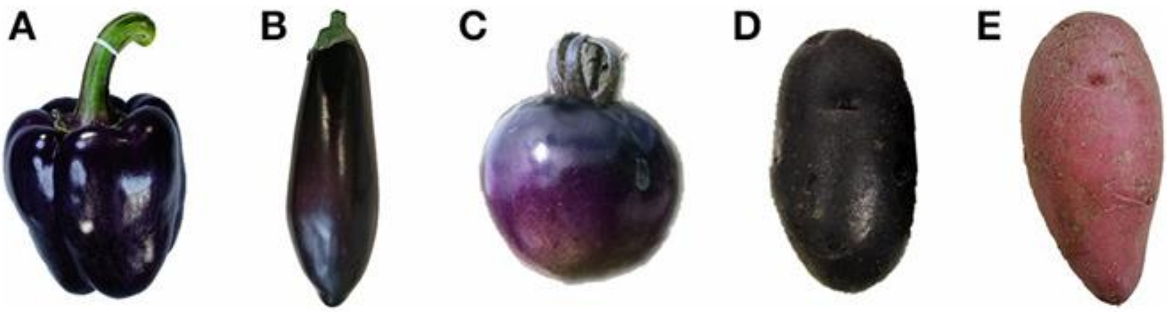
In a post-harvest study of pepper fruits, severe losses are caused by a necrotrophic fungus called *Botrytis cinerea* (Wang et al., 2022). It is found in that when a green pepper is inoculated with the pathogen the fruits were found to be severely rotten after 1 week. The area of inoculation has undergone chlorosis, turned brown and soft with grown layers of grey mold.

It has been observed in the studies of (Z. Zhang et al., 2015), that anthocyanins often act as the first line defence against the pathogen attacks. When the accumulation of anthocyanin had been reduced in tomatoes, the areas with less anthocyanin were more susceptible to *Botrytis cinerea* than the purple areas (Zhang et al., 2015).

2.3. Anthocyanins

Anthocyanins are an important class of flavonoids which represent a large class of secondary metabolites of plants. Anthocyanins are glycosylated polyphenolic compounds also known as conspicuous class as they have a wide range of colours varying from orange, red and purple to blue in flowers, seeds, fruits and vegetative tissues (Holton' & Cornish, 1995); Liu et al., 2018). These plant pigments are also abundant in several members of the nightshade family (fig. 4.). Anthocyanins are mostly present in the cell vacuoles as they are water soluble pigments and thereafter their hue, a colour property influenced by the intravacuolar environment. 600 estimated anthocyanins are found in nature, and they are expecting to find more (Smeriglio et al., 2016). The six common anthocyanidins pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin are the precursors of the most prevalent anthocyanins found in plants (Liu et al., 2018).

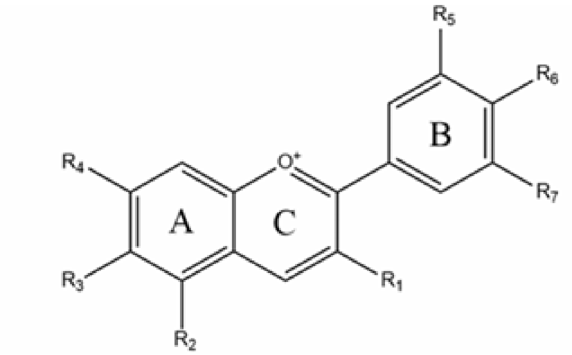
Figure 4. - Example of Solanaceous vegetables rich in anthocyanins. (A) purple pepper fruit, (B) purple eggplant fruit, (C) purple tomato fruit, (D) purple potato tuber, (E) red potato tuber (Liu et al., 2018).



Anthocyanins are composed of a backbone called anthocyanidin with sugar and acyl conjugates. Anthocyanidins are formed of two benzene rings which are separated by an oxygenated heterocycle (Liu et al., 2018). Anthocyanidins are important class of flavonoids which are of medium size and are coloured. Out of them 25 different types of anthocyanidins are known where each one is different from each other with the presence of hydroxyl and methoxy groups bound at the core of the molecule. Eventually, anthocyanidins are grouped into 3-hydroxyanthocyanidins, 3-deoxyanthocyanidins, and O-methylated anthocyanidins. Cyanidin, Delphinidin and Pelargonidin are among the three non-methylated anthocyanidins, that are the most common in nature. In most of the cases, anthocyanidins are bounded with a sugar moiety to form the corresponding anthocyanins (Mannino et al., 2021).

After the sugar part is added at the third and/or fifth position (R1 and/or R2 subsistent of the chemical structure shown in fig. 5.), glycosylation is accomplished enzymatically. Compared to their related anthocyanidins, anthocyanins are more stable and soluble in water as a result of glycosylation (Mannino et al., 2021).

Figure 5: Chemical scaffold of anthocyanin compounds and their relative substituents (Mannino et al., 2021)



2.3.1. Biosynthesis

Anthocyanidins and anthocyanins can be produced by plants exclusively, a branch of the phenylpropanoid pathway which is also involved in the other flavonoid synthesis (Mannino et al., 2021). The anthocyanin biosynthetic pathway is an extension of flavonoid pathway (Liu et al., 2018) as shown in the fig 6. The enzymes responsible for the synthesis of anthocyanidins are settled as a multi-enzyme complex in the endoplasmic reticulum, structured as a multi-enzyme complex called as the flavonoid metabolon (Tanaka et al., 2008). According to (Holton' & Cornish, 1995) all the flavonoids including anthocyanins are synthesized form malonyl-CoA and p-coumaroyl-CoA. Three enzymes are required to covert the colourless dihydroflavonols to anthocyanins (Holton' & Cornish, 1995). At the first point of the path, phenylalanine is transformed into cinnamic acid by phenylalanine ammonia-lyase (PAL), which is subsequently converted into coumaric acid by cinnamic acid 4-hydroxylase (C4H). Upon being activated by the 4-coumarate-CoA ligase (4CL), 4-coumaryl-CoA combines with three molecules of malonyl-CoA in a process mediated by chalcone synthase. This reaction results in the creation of 4-hydroxychalcone (e.g., naringenin chalcone) and marks the beginning of the flavonoid biosynthesis pathway. Chalcone isomerase (CHI) transforms the 4-hydroxychalcone into the corresponding 7,3',5', trihydroxyl-flavone (such as naringenin). Following this, 7,3',5', trihydroxyl-flavone is oxidized by flavanone 3-hydroxylase (F3H) to produce flavanol-form (such as dihydrokaempferol). Then, flavonoid 3'-hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H) convert dihydrodrokaempferol into either dihydromyricetin or dihydroquercetin. Dihydroflavonol-4-reductase (DFR) and anthocyanidin synthase (ANS) must work together to transform the three hydroflavonols into anthocyanidins. The leucoanthocyanidins are formed by the first enzyme, whereas each leucoanthocyanidin is oxidized into 2-flavan-3,4-diol by the second enzyme, which is reliant on 2-oxoglutarate. These later substances transform into the corresponding anthocyanidins on their own (Mannino et al., 2021).

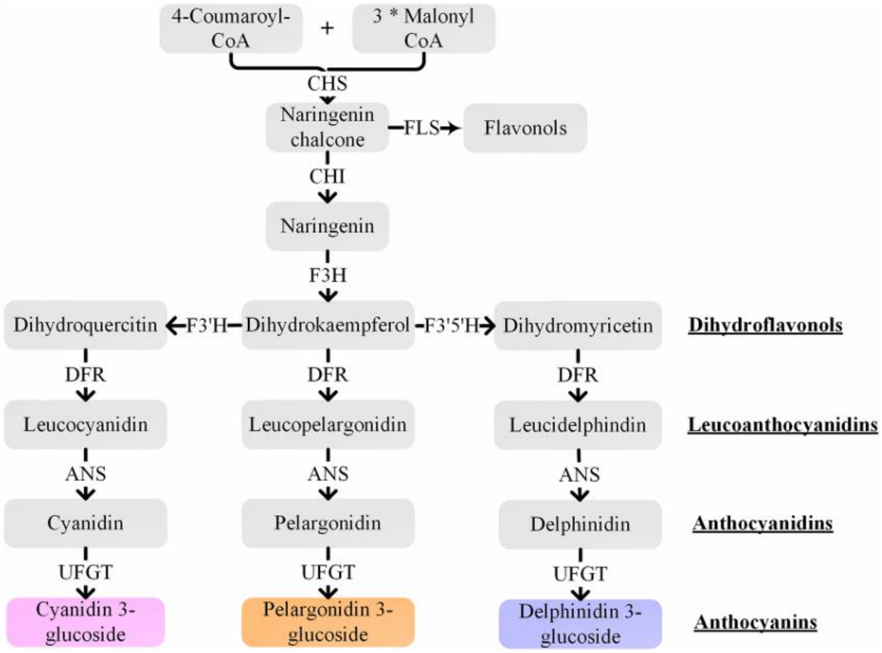
Anthocyanins are non-photosynthetic pigments produced in the cytoplasm and retained in the vacuolar lumen of epidermal cells (Nassour et al., 2020).

Different types of anthocyanin accumulation were seen within the main vacuole, and their variety was reflected in the many names used to describe them. Despite the majority of cells have anthocyanin vacuolar sap that is uniformly coloured, several instances of regular or irregularly shaped pigmented bodies known as anthocyanin vacuolar inclusions have recently been documented (Nassour et al., 2020).

Anthocyanins are glycosides of anthocyanidins. A sugar moiety of anthocyanin is acylated with aliphatic and aromatic acids. Thus, even though the number of known anthocyanidins are 25, there are several hundreds of derivatives can be identified because of the diversity offered by glycosylation and acylation (Mortensen, 2006).

Because of their instability, anthocyanidins are not commonly discovered in nature as free aglycones; rather, they are referred to as anthocyanins after they have been glycosylated with one or more sugar molecules (Nassour et al., 2020).

Figure 6: Representation of the anthocyanin biosynthetic pathway (C. Ma et al., 2018)



The Colour of Anthocyanins: The number of hydroxyl and methoxyl groups in anthocyanins' structure determines their colour intensity and kind. The colour tends to be bluer if it has more hydroxyl groups (OH). However, if it contains more methoxyl groups (OCH3), the redness rises. Because anthocyanins are ionic, their colour also varies with pH. Certain anthocyanins turn red in acidic environments. When the pH is at neutral, they are purple, but when the pH rises, they turn blue (Nassour et al., 2020).

Because of their unique molecular structure, anthocyanins and anthocyanidins have stronger antioxidant properties than other flavonoids. These chemicals' ability to reduce metal-induced peroxidation can be ascribed to their ability to bind metal ions that are implicated in the generation of free radicals (Nassour et al., 2020).

2.3.1. Anthocyanin Function

Anthocyanins act as strong antioxidants, by preventing the lipid peroxidation and oxidative damage by scavenging the oxygen radicals by donating a hydrogen atom from their hydroxyl group or electrons and neutralize the radicals (Gould et al., 2002). Due to their higher antioxidant properties, they protect plants from different biotic and abiotic stresses. Anthocyanins' antioxidant activity is contingent upon the kind and degree of acylation and glycosylation, as well as the degree of hydroxylation at the B-ring. Hydroxylation at the B-ring accelerates the antioxidant capacity(−OH>−OCH3>>−H) and thus the antioxidant capacity of anthocyanins is in this order of delphinidin>petunidin>malvidin=cyanidin. The antioxidant capacity decreases as the sugar units at C3 and C5 position increases (Liu et al., 2018).

Enzymatic activity of Anthocyanins:

Anthocyanins are known for their antioxidant activity by playing an important role in protecting the plant tissue against oxidative stress. The antioxidant properties of anthocyanins can scavenge the reactive oxygen species (ROS) such as hydrogen peroxide and superoxide radicals and in result reducing the oxidative damage in calls (Sadowska-Bartosz & Bartosz, 2024)

2.3.2. Anthocyanin Benefits

Anthocyanins are red pigments that protect plants from stressors like high light, UV, drought, and herbivory by scavenging reactive oxygen species and shielding photolabile compounds. They enhance nitrogen resorption, improve stress tolerance, and support plant survival. Their significant metabolic cost highlights their essential functional role rather than being a by-product of flavonoid biosynthesis (Gould, 2004). Anthocyanins are crucial for plant reproduction because they provide vibrant colours which attract pollinators and seed dispersers (J B Harborne, 2000). Anthocyanins offer plants their vibrant characteristics while also protecting them from a variety of biotic and abiotic stressors (Chalker-Scott, 1999), which could assist them adapt to climate change more effectively (Liu et al., 2018). The photosynthetic equipment is shaded and protected by anthocyanins, which are photoprotective chemicals that scavenge free radicals and absorb excess visible and UV radiation (Kevin S. Gould, 2004). For example, compared to tissues without anthocyanins in red pear fruits and purple pepper leaves with anthocyanins exhibited a more consistent PS II photosynthetic capability and a better resistance to photo-oxidation (Pengmin Li, 2008). Moreover, anthocyanins frequently build up in the sun-exposed side of fruits and young leaves to shield them from photoinhibition and photobleaching under light stress without seriously impairing photosynthesis [(P. Li & Cheng, 2008); (Zhu et al., 2018)]. A Study says that anthocyanin acts as a sunscreen by absorbing visible light particularly in green wavelengths and therefore reduce the light stress on photosynthetic tissues (Manetas & Buschmann, 2011).

Additionally, the presence of coloured anthocyanins could reduce disease and insect infestation. In this case, the *Helicoverpa armigera* larvae failed to select tobacco leaves packed with anthocyanins. Compared to controls given green leaves, feeding anthocyanin-pigmented leaves considerably increased the mortality of *H. armigera* larvae and substantially delayed the pupation of *Spodoptera litura* (Malone et al., 2009). Post harvest tomato fruits which are rich with anthocyanins displayed less vulnerability to gray mold (*Botrytis cinerea*), delayed over ripening and therefore doubles the shelf life (Y. Zhang et al., 2013) . Additionally, transgenic tomato plants exhibited improved resistance to heat stress when their anthocyanin content was higher (Xia Meng, 2015). Anthocyanin-rich leaf tissue that had been injured recovered more quickly from mechanically induced oxidative stress (Gould et al., 2002).

In addition to playing important roles in plant and microbial signalling, anthocyanins are also antimicrobials feeding deterrents, and the cause of male fertility in some species (Simcha Lev-Yadun, 2008). They are crucial for biological defence, photoprotection, and the symbiotic relationships between plant cells and microorganisms. There is mounting evidence that anthocyanins play a function in the physiological survival of plants under different abiotic stresses, especially when they are found in the epidermal cells or on the top surface of the leaf (Nassour et al., 2020).

Anthocyanins also play a major role during the cold stress. At low temperatures, in plants there is a reduction of membrane fluidity, enzyme activities which includes functions like photosynthesis. And eventually the photosynthetic rate decreases which will promote ROS production which can result in cell damage. Low temperatures enhance the production of anthocyanins by up regulating the anthocyanin biosynthetic genes which in turn increase anthocyanin accumulation. As anthocyanins have antioxidant capacity, they can reduce the oxidative stress (Nassour et al., 2020). A Study proved that lower temperature have been shown to enhance anthocyanin accumulation in purple head chinese cabbage seedling (He et al., 2020)

Besides providing protection to the plant during its growth, anthocyanins also play an important role to improve the postharvest performance of vegetables. For instance, anthocyanins being the antioxidants they can prevent lipid peroxidation and can maintain membrane integrity to slowdown the death of the cell (Liu et al., 2018).

Shelf life is one of the most critical quality traits of the food, especially for the fleshy fruits like tomato. Tomato's shelf life is usually short due to its fast over- ripening which is caused by several factors like change in temperature, respiration and pathogen exposure. Tomatoes have a very less amount of anthocyanin content, but varieties which are enriched with antioxidants have been developed. Those tomatoes which contain anthocyanin has shown to acquire a quality to extend the shelf life significantly by delaying the over ripening and therefore resulting in being less susceptible to pathogens (Y. Zhang et al., 2013) (Petric et al., 2018).

Anthocyanins usually appear red in the leaf cells, but depending on their chemical nature, concentration, the pH, and interaction with other pigments can result in pink, purple, blue, orange, brown and even black leaf colours. The specifics of how insects without red photoreceptors perceive red colours are yet unknown. Some aphid species may exhibit colour discrimination, which might be explained by a colour opposition mechanism. This calls for positive excitement in the green waveband and negative excitation in the blue and ultraviolet. Nevertheless, it is unclear how such a process can make it easier to perceive anthocyanin leaves, which normally reflect less UV and green light than green leaves. It's possible that insect herbivores find red leaves less appealing because they stimulate their green receptor more slowly than they do when they are aroused by green leaves. Red leaf colour and/or brightness contrasts with the surrounding visual backdrop are probably also significant (Lev-Yadun & Gould, 2008).

2.3.3. Effect of Light on Anthocyanin Biosynthesis

One of the most substantial environmental elements impacting the accumulation of anthocyanins is light. Many plant species produce more anthocyanins when exposed to high light levels (Trojak & Skowron, 2017). For instance, when the surface of tomato fruit is directly exposed to light, it expressed a more intense anthocyanin accumulation than the shaded parts of the plant (J. Li et al., 2025). Some studies suggest that UV-A radiation on tomato fruits has better effect on anthocyanin development than in the normal white light. Blue and red light can also induce anthocyanin pigmentation compared to darkness (Liu et al., 2018).

Plants have evolved advanced photoreceptor systems, including phytochromes (red/far-red photoreceptors), cryptochromes, phototropin (blue/UV-A photoreceptors), and UVR8 (UV-B photoreceptors), to acclimate fluctuating light conditions. Photoreceptors detect varying light conditions, leading to structural alterations that activate receptor proteins, which then translocate to the nucleus to engage with positive transcription factors or inhibit the master negative regulator COP1 (constitutively photomorphogenic 1), which normally is active during the dark. In the light when COP1 is inactive, it allows HY5 (ELONGATED HYPOCOTYL 5, and it is a master transcription factor in plants) to accumulate and initiate the anthocyanin biosynthesis genes like MYBs (transcription factor which turns on anthocyanin related genes) and CHS/DFR/ANS (enzymes which catalyse the synthesis process). Blue light can specifically activate anthocyanin production responsible module whereas red light has a weaker effect (Y. Ma et al., 2021).

Through the enhancement of anthocyanin production, a rise in the blue light percentage raised anthocyanin levels. Kinetic modelling and increased levels of expression for the anthocyanin biosynthesis genes *CaMYB, CaCHS, CaDFR, CaANS*, and *CaUFGT* support this. As the fraction of blue light increased, the anthocyanin biosynthesis pathway was strengthened, as evidenced by the higher expression levels of several anthocyanin biosynthesis genes (*CaMYB, CaDFR, CaANS,* and *CaUFGT*) in B-72% and B-99% fruit compared to B-24% fruit (Liu et al., 2022). These studies also demonstrated that pepper fruit showed higher levels of anthocyanins when exposed to higher fractions of blue light, particularly B-72%, than when exposed to B-24%. Anthocyanin content may also be influenced by the light spectrum. Compared to white or red light, blue light both increased anthocyanin content and the transcript level of anthocyanin biosynthesis genes. Degradation of anthocyanins was noted in purple *Capsicum annuum* genotype fruit. Several ripening-related processes in pepper fruit were slowed by higher blue light fractions. This suggested that spectrum has a greater impact on pepper fruit ripening than intensity (Liu et al., 2022).

A study showed that delphinium 3-O-glucoside biosynthesis in light exposed pepper has significantly increased after 48 hours compared to the shaded surface of the pepper fruit.(Yan Zhou, 2022)

2.3.4. DNA Methylation

DNA methylation is a significant epigenetic phenomenon that adds methyl groups to cytosine bases, especially in promoter regions, to control gene expression. Demethylation has the ability to promote transcription, whereas methylation frequently inhibits gene activity. In sweet orange (*Citrus sinensis* L.), showed that anthocyanin formation during cold stress was closely associated with alterations in DNA methylation. By employing quantitative PCR and the methylation-sensitive endonuclease McrBC, they discovered that demethylating promoter regions of important anthocyanin-related genes, such DFR and Ruby, enhanced anthocyanin production and gene expression. This implied that alterations in DNA methylation status controlled, at least in part, the development of citrus pigments brought on by cold (Sicilia et al., 2020).

The effects of light exposure following bag removal on anthocyanin accumulation in non-red apple cultivars like ‘Granny Smith’ and ‘Golden Delicious’ are being studied. They found that the expression of the transcription factor MdMYB1 was strongly correlated with the intensity of the colour and identified it as a key regulator of anthocyanin production. The study found that light exposure decreased methylation levels in particular promoter regions of MdMYB1 (−2026 to −1870 bp, −1898 to −1633 bp, and −541 to −435 bp), leading to increased gene expression and red colouring, according to bisulphite sequencing. ‘Golden Delicious’, on the other hand, continued to have more promoter methylation, which resulted in lower amounts of MdMYB1 transcript and poorer colouring (Sicilia et al., 2020).

Additionally, the same study demonstrated that demethylation may be generated experimentally. When ‘Granny Smith’ apples were treated with the chemical demethylating agent 5-aza-2′-deoxycytidine, their anthocyanin concentration and red colour were greatly increased. The MdMYB1 promoter's methylation was significantly reduced as a result of this treatment, and structural genes involved in the anthocyanin biosynthesis pathway—including *CHS, DFR, ANS*, and *UFGT*—were expressed at higher levels. These results showed that light may cause demethylation to increase pigment accumulation and that promoter methylation directly affects the activation of genes involved in anthocyanin production (C. Ma et al., 2018).

In summary, these investigations demonstrate that DNA methylation plays a conserved function in regulating the production of anthocyanins in response to environmental stimuli. Cold stress in citrus caused anthocyanin-related genes to become activated and demethylated (Sicilia et al., 2020). However, the MdMYB1 promoter in apples was similarly affected by light exposure (C. Ma et al., 2018).

2. MATERIALS AND METHODS

3.1 Plant Materials and Experimental Design

The plants that are used in this research are originating from the cross of ‘Fehérözön’, which is a Hungarian cultivar and a ‘Black pearl’ a deep purple cultivar, the plant material studied is from the F2 segregating generation.

For the studies 5 plants were selected. These 5 samples were sampled at week 0 and 2 leaves of each were then covered with tin foils (fig 7). After covering the plants, we let it grow in the light. And after 10 days, the leaves are collected again.

Figure 7. - Pepper plants with exposed leaves and covered leaves (Source: own work)

3.2. Analytical measurements

After the collection of leaves, the samples were measured and grounded using a mortar and pestle while adding liquid nitrogen and mixed with a solvent mixture (60:39:1%; 60 mL methanol 39 mL distilled water, 1mL formic acid). And the mixture is subjected to centrifuge to separate the solid and liquid components. The supernatant was stored at -20 degrees Celsius for further analysis.

3.2.1 Total Polyphenolic content

The total polyphenolic content (TPC) was measured with Folin–Ciocalteu reagent according to Singleton and Rossi (1965), at λ = 760 nm with a Hitachi U-2900 spectrophotometer (Aryal et al., 2019a)

- 50 mL Folin + 500 mL distilled water
- 80:20: methanol: distilled water (MeOH:DW) – solvent
- Na_2CO_3 – 37.1 grams in 500 mL Distilled water
- 0.056 grams of Gallic acid (3 mM concentration) to 100 mL of 80:20 MeOH:DW solvent. 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, as shown in the table 1. (Aryal et al., 2019).

Table 1.: Calibration protocol **(**source: own work)

SI	**Folin** (µl)	**MeoH:DW** (µl)	**Galic acid** (µl)	**Na2CO3** (µl)
1	1250	250	0	1000
2	1250	250	0	1000
3	1250	200	50	1000
4	1250	150	100	1000
5	1250	100	150	1000
6	1250	50	200	1000
7	1250	0	250	1000

➔ **Sample Measurements**

Samples are measured in triplicates, 10 µL extract was applied for assay. The measurements were carried out using a Hitachi U-2900 spectrophotometer.

3.2.2 Ferric Reducing Abilities of Plasma (FRAP) – antioxidant capacity

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 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.

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 (Gougoulias et al., 2017) And the measurements were taken as shown in the table 2.

Table 2.: Calibration protocol **(**source: own work)

FeSO4 µL	**Water µL**	**Working solution µL**	**Mmol/L**
0	66.6	2000	0
3.3	63.3	2000	6
13.3	53.3	2000	12
26.6	39.9	2000	18
39.9	26.6	2000	24
53.3	13.3	2000	32
66.6	0	2000	36

➔ **Sample Measurements**

Samples were measured in triplicates following the protocol from table 3.2.2, 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.

3.2.3 Total Flavonoid Content (TFC)

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) as shown in the protocol table 3.2.3, thus the results were expressed in mg quercetin equivalent per 1 g fresh weight (Adefegha & Oboh, 2011).

Required materials:

- 95% EtOH (ethyl alcohol) – 375 µl
- 10% Aluminium chloride – 25 µl
- 1 M Potassium Acetate – 25 µl
- Distilled water – 700 µl

To the above solution, I added 100 µl sample and let it incubate for 30 min.

➔ **CALIBRATION:**

For the calibration we need quercetin stock which can be made 200 µg/ml (EtOH).

Table 3.: Calibration protocol (source: own work)

Quercetin (polyphenol)	**Distilled water**	**µg/ml**
0	100	0
10	90	20
20	80	40
40	60	80
60	40	120
80	20	160
100	0	180

3.3 DNA Methylation Analysis

In this work, the impact of light on anthocyanin production and DNA methylation in leaves was examined using pepper (*Capsicum annuum*) plants. For this the same set of five plants were applied described in chapter 4.1. DNA isolation from the illuminated and covered leaves are done using the Macherey-Nagel Nucleospin Plant II kit according to the following protocol (GmbH, 2014).

3.3.1. DNA digestion

In the first step, the samples were digested in separate reactions with the *Msp*I and *Hpa*II 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*I or *Hpa*II, 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 4 were used.

Table 4.: Sequences of adapters, pre-selective, and selective primers (Reyna-López et al., 1996).

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

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

3.4. Detached leaf assay

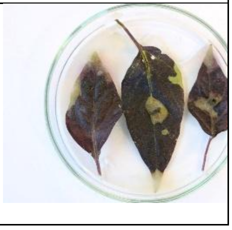
The detached leaf assay was done on the same F2 plants. For the study green and purple leaves were taken off from the plants and sterilized in sodium hypochlorite for 20 minutes. The sterilized leaves were then placed onto Murashige and Skoog media under sterile conditions in a laminar air flow. The inoculation of the leaves was done with the agar slice method. For these 10 days old *Botrytic cinerea* cultures grown on potato dextrose agar (PDA) media were used, agar slices were cut out from the *Botrytis* 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 4 days as shown in the figures 8 and 9.

Figure 8. - *Botrytic cinerea* inoculated on green leaves (Source: own work).

Day 1	Day 2	Day 3	Day 4
			
			
			
			

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.

Figure 9. - *Botrytis cinerea* inoculated on purple leaves (Source: own work).

Day 1	Day 2	Day 3	Day 4
			
			
			
			

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 90°C 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 analysed 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 $\lambda = 460$ 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.

3. RESULTS AND DISCUSSION

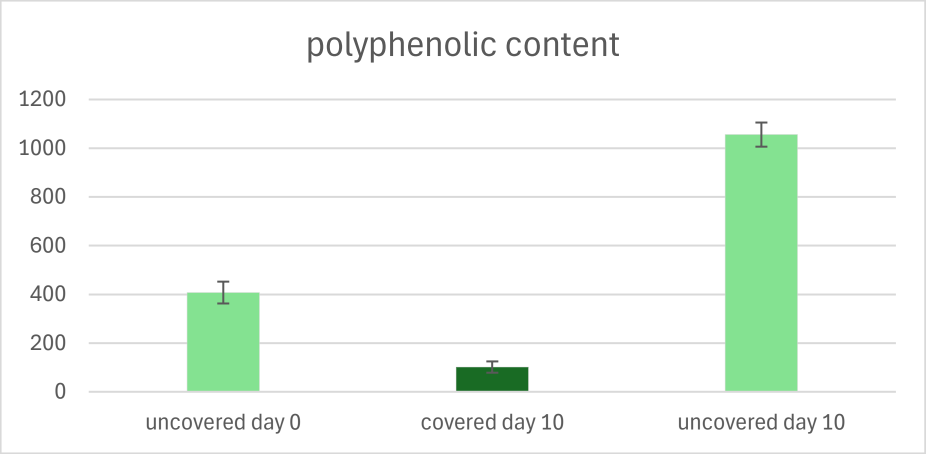
The experimental results from the examination of *Capsicum annuum* F_2 plants exposed to different light conditions are shown in this section. The main goal of these experiments was to figure out how the light exposure affects anthocyanin, antioxidant capacity and DNA methylation patters in pepper leaves. To achieve this a comparison strategy was adopted with leaves exposed to light vs leaves restricted to light. The analytical measurements (TPC, TFC, FRAP, and TMA) were used to analyse pigment and antioxidant levels, while MSAP analysis was applied as a genetic and epigenetic measurement used to determine possible methylation changes between light and dark treatments. The following findings describe the observed differences and statistical connections generated from these measures.

4.1 Analytical measurements

4.1.1 Total polyphenolic content

Polyphenols are secondary metabolites with strong antioxidant activity by performing protective roles against environmental stress. Calculating the total polyphenolic content provides an estimate of the leaf's overall antioxidant potential. In this study, TPC was measured using the Folin-Ciocalteu technique and represented as mg gallic acid equivalent (GAE) per gram fresh weight. This study examines how light exposure impacts the polyphenol accumulation in *Capsicum annuum* leaves.

Figure 10. - TPC in light exposed and dark treated leaves of *Capsicum annuum* (Source: own work)

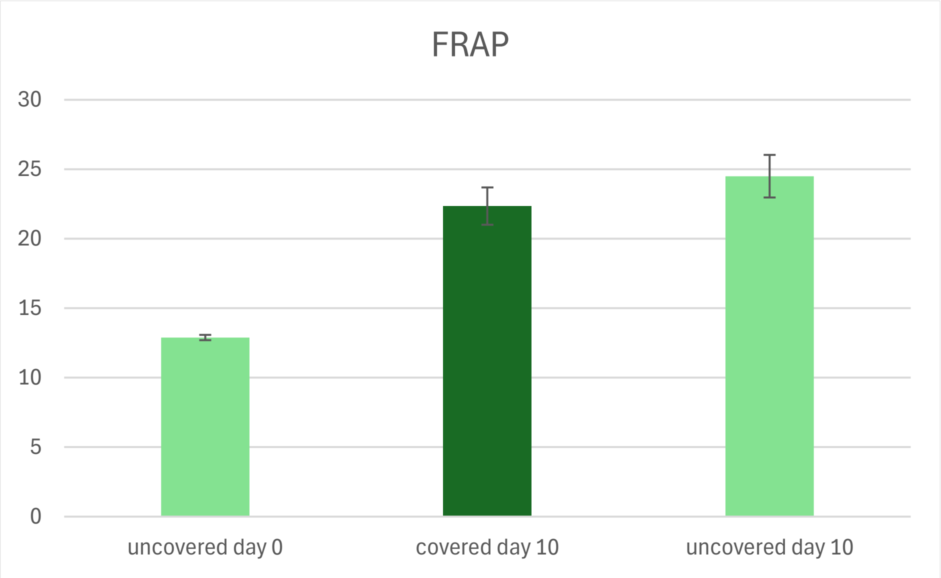


Based on the results (fig 10.), the light exposed leaves have a higher TPC value than the leaves that were covered and remained in dark. In the graph day 10 uncovered which is light exposed showed a TPC content of 1055.8711 mmGS/g and the day 10 covered which was left in dark conditions showed 101.5144 mmGS/g. The polyphenolic content is almost 10 times higher in day 10 Uncovered leaves than day10 covered leaves. We can also observe that day 0 TPC content which is 407.4677207. This shows the comparison between covered and uncovered leaves from day 0 to day 10. This increase in TPC under light conditions showed that the light stimulates the production of polyphenolic compounds, and therefore increasing the plants antioxidant defence. Similar study was done, and the results were reported by (Aryal et al., 2019) who noted variances in the polyphenolic and flavonoid content of wild edible green vegetables and attributed these variations to habitat and light intensity.

4.1.2 Ferric Reducing Ability of Plasma (FRAP)

The FRAP assay measures the ability of antioxidants in a sample to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) ions. This technique was first created for plasma samples; however, it is frequently used to determine the reducing power of plant tissues. Higher FRAP value indicates that a sample has stronger ability to reduce Fe^{3+}, which implies a higher antioxidant capacity.

Figure 11. - Ferric Reducing Antioxidant Power (FRAP) of light exposed and dark treated leaves of *Capsicum annuum* (Source: own work).

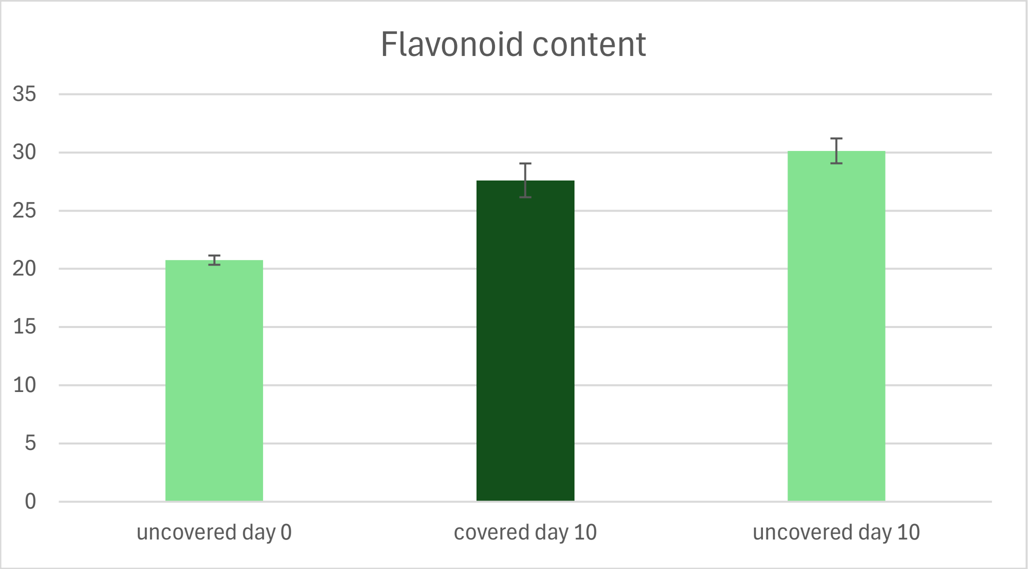


In this study, based on the results from fig 11, the light exposed leaves samples showed a higher FRAP value when compared to the dark treated. In case of day 10 uncovered the frap content is 22.3493 µmAS/g and day10 covered showed a FRAP content of 24.5007 µmAS/g (These samples are from the leaves of sample plant collected on the same day); there is a noticeable difference. This suggests that light exposure increases the antioxidant capacity of *Capsicum annuum* leaves most likely due to the enhanced production of polyphenolic and flavonoid chemicals. (Góra & Csepregi, 2024) discovered a similar trend reporting increased FRAP values in pepper leaves exposed to UV radiation compared to control plants cultivated under UV-blocking circumstances. The similar result among these data suggests that increasing light or UV exposure increases the creation of secondary metabolites.

4.1.3 Total Flavonoid Content (TFC)

Flavonoids are a class of plant secondary metabolites that exhibit antioxidant, anti-inflammatory, and antibacterial effects. Measuring their total flavonoid content in leaves reveals the plant's bioactive potential. Higher flavonoid concentration is generally associated with increased antioxidant activity.

Figure 12. - Total flavonoid content in light exposed and dark treated leaves of *Capsicum annuum* (Source: own work)

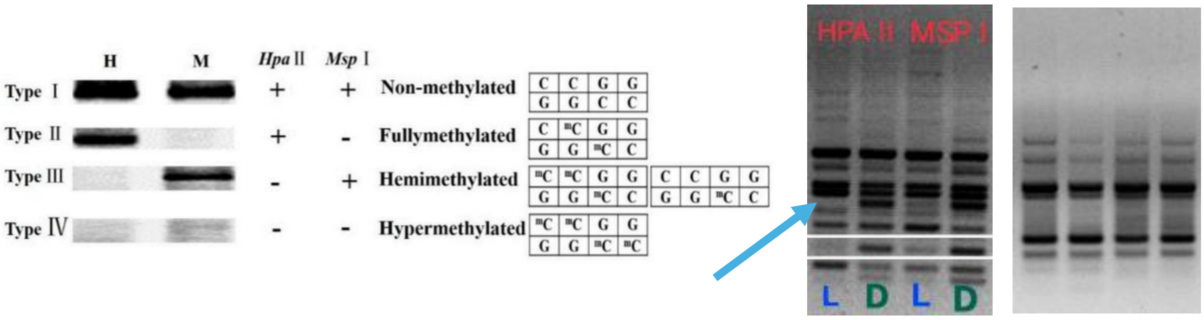


Based on the results (fig 12), the total flavonoid content of *Capsicum annuum* leaves was greater in case of day 10 uncovered leaves which is o30.1351 mmQ/g than the day 10 covered leaves which is 27.6032 mmQ/g. The graph also compares between day 0 which is 20.74 mmQ/g to day 10 of uncovered leaves (30.1351 mmQ/g) and there is an increase in TFC content It is safe to say that light exposure most likely stimulates the flavonoid production as a defensive response. Because of the lack of light, the covered leaves showed a lower flavonoid content. These results suggest that light is an important regulator for flavonoid synthesis, increasing the antioxidant capacity of the leaves. (A. Li et al., 2016) reported similar findings, discovering that *Lithocarpus litseifolius* cultivated under higher light intensities accumulated considerably higher flavonoid levels than plants grown under severe shade. They attributed this to light-induced stimulation of flavonoid biosynthetic pathways, which protect leaf tissues from photooxidative damage.

4.2 Methylation sensitive marker method

I used MSAP method where it compares the digestion by two enzymes such as *Hpa*II and *Msp*I. *Hpa*II and *Msp*I, two enzymes that both recognize CCGG but cut differently depending on methylation, are used in a methylation-sensitive marker method to compare digestion. A band ("+") on our gels indicates that the enzyme cut and that the site is unmethylated at the appropriate cytosine; a band ("−") indicates that methylation prevented cutting.

Figure 13. - Four methylation types based on banding patterns, methylation pattern of the illuminated (L) and covered (D) leaves, run on a 2% TBE EtBr stained agarose gel, differences are marked with arrow or brackets (Source: own work).



In fig 13, on the gel photo highlighted with the blue arrow, it can be seen that in the L (illuminated) sample the *Hpa*II could not digest the DNA, whereas the *Msp*I could (-, + indicates hemimethylation), while the covered sample could be digested by both of the enzymes (+, + indicates a non-methylated region). Thus, it went from hemimethylated to non- methylated, these differences were gathered and displayed in Table 5.

Table 5. – Differences in the methylation pattern caused by the different light (Source: own work).

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

As shown in the table 5, we compare the methylation patterns between leaves that are grown in light (L) and dark (D) to understand epigenetic regulation of anthocyanin. As shown in the fig 12. there are 4 methylation band patterns. These methylation types shift when the light and dark samples are compared, suggesting that methylation status varies depending on the growth circumstances.

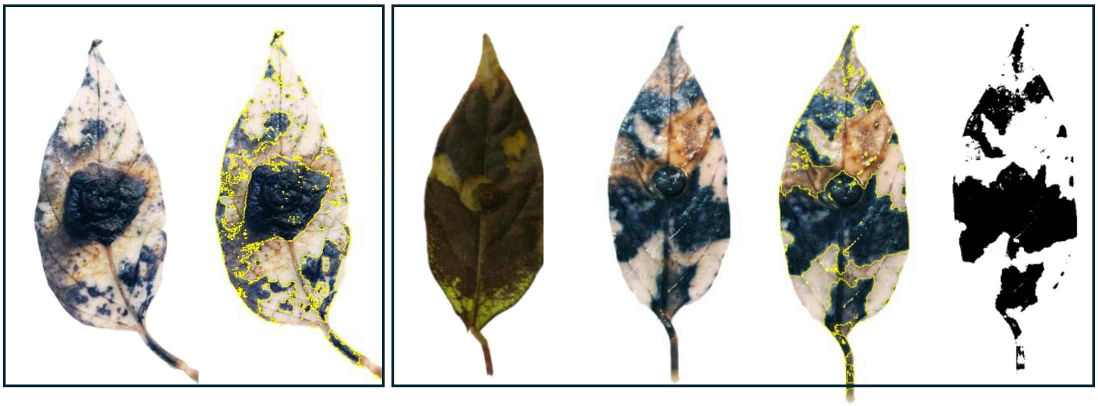
In this analysis, we found that locus specific methylation shifts between light and dark leaves, most differences occurred in the Type I category, where in the light the samples were not methylated but in the dark, they became either fully or hemi methylated (Table 5.). Many loci become methylated in the dark, which is connected with the suppression of genes involved in the anthocyanin pathway. This supports the theory in which darkness promotes methylation-linked silencing while light keeps the chromatin state at pigment-associated loci more open (less methylated). For example, type I in the presence of light changed to Types II, III, or IV in the absence of light. These transitions, which involve both partial (Types II and III) and full (Type IV) methylation states, show an overall increase in methylation frequency when plants were grown in darkness. (Omidvar & Fellner, 2015), in this study they observed a similar pattern in seedlings of Solanum lycopersicum ('7B-1' male-sterile line), where MSAP profiles revealed higher methylation under dark (D) compared to light (white, blue, red) growth conditions, and several differentially methylated fragments were associated with anthocyanidin biosynthesis.

These similarities support the idea that light exposure may keep loci in a less methylated (active) state, allowing for the biosynthesis of anthocyanins, darkness might encourage methylation-linked silencing of pigment-associated genes.

4.3. Detached leaf assay

The detached leaf assay was used to determine how susceptible the green and purple leaves of *Capsicum annuum* plants were to infect by *Botrytis cinerea.* The inoculated sites were surrounded by visible necrotic lesions after four days of incubation: the severity of the infection varied depending on the kind of leaf. In order to determine the percentage of infection, ImageJ analysis was utilized to quantify the infected area in relation to the overall leaf area.

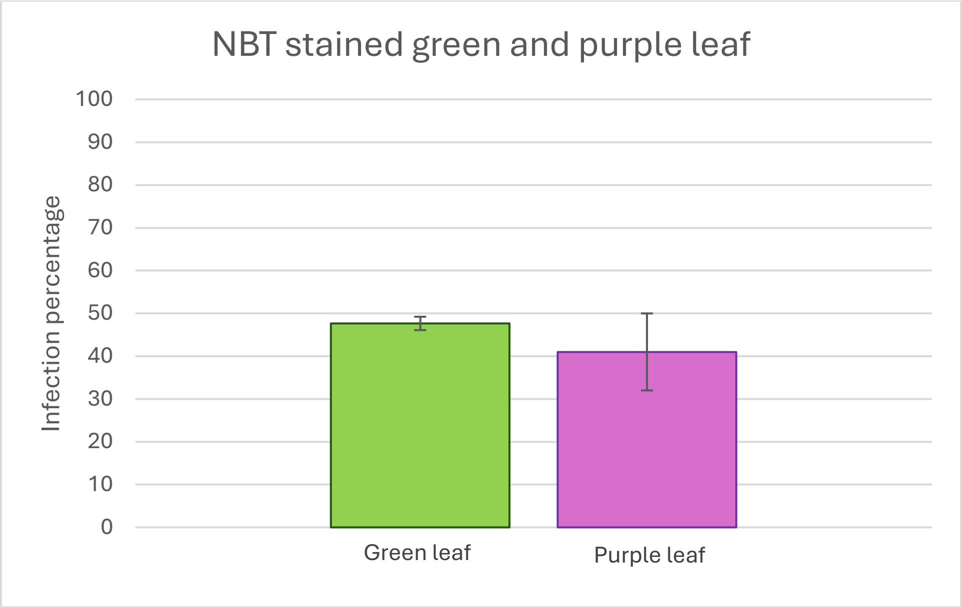
Figure 14. - Green leaf (to the left) and purple leaves (to the right) after staining with nitro blue tetrazolium chloride (NBT) and were processed with Image J software (Source: own work)



The leaves are treated with NBT (nitro blue tetrazolium chloride) as it is used to detect the superoxide radicals in plant tissues. These superoxide radicals release as a response to stress and infection can be the bigger trigger. NBT reacts with superoxide radicals and produce blue compound. Therefore, NBT staining can allows to visualize the super radical production and therefor the infection clearly (Kuźniak et al., 2014). In the fig 14 we can observe that in the purple leaf before staining it looked normal, but when it is stained with NBT we can see the blue compound which implies infection in it. To calculate the amount of superoxide produced, the stained area was measured with the help of ImageJ, for this only the blue coloured sections were selected and then converted to black and white as shown on fig 14. Figure 15 illustrates that *Botrytis cinerea* infection was more severe in green leaves compared to the purple leaves.

This indicates that presence of anthocyanin can reduce the levels of infection. These results imply that anthocyanin pigments in purple leaves defend against *Botrytis cinerea* infection.

Figure 15. - Graph showing the comparison of infection on green and purple leaves (Source: own work)



Diaminobenzidine (DAB) staining is a histochemical method which is used to detect peroxide (H_2O_2) which is one of the reactive oxygen species (ROS) produced in plants during stress or pathogen attack. DAB is oxidized by H_2O_2 in the presence of heme containing proteins like peroxidases and they generate a dark brown colour (Obrien, 2012).

The brown staining appears on the leaves where the pathogen infection is harsher such as shown in the fig 16. For the calculation the same ImageJ method was used with the brown spots selected and converted to a binary black and white image and the area measured (fig 16.).

Figure 16. – DAB stained green leaves to the left and purple leaves to the right (Source: own work)

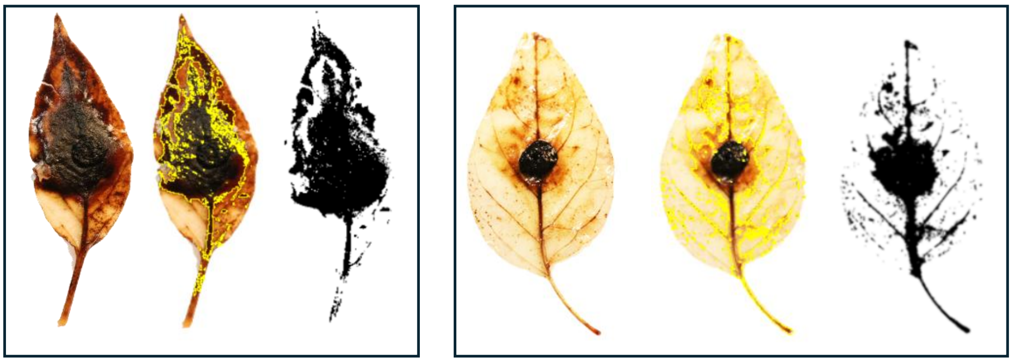
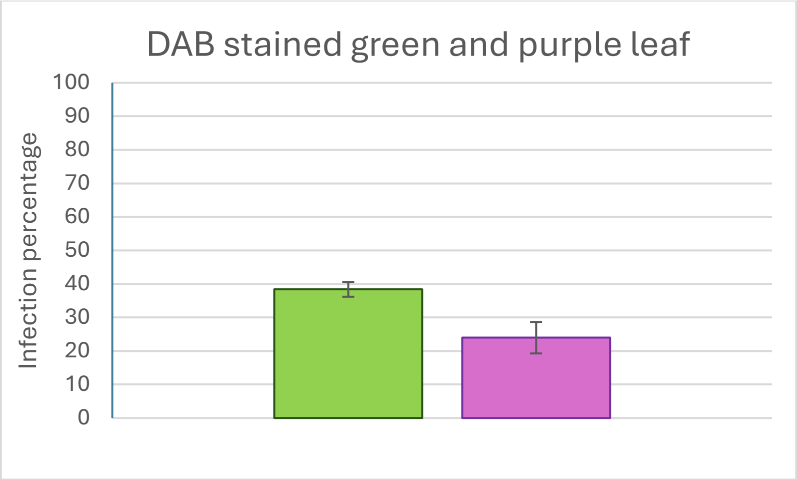
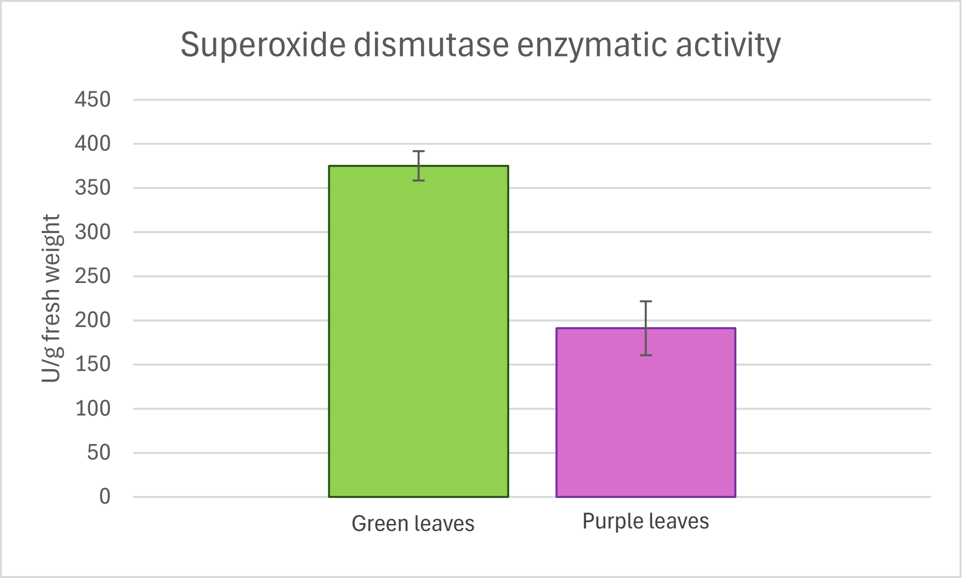


Figure 17. – Comparison of DAB stained leaves infection percentage of the green and purple leaves (Source: Own work)



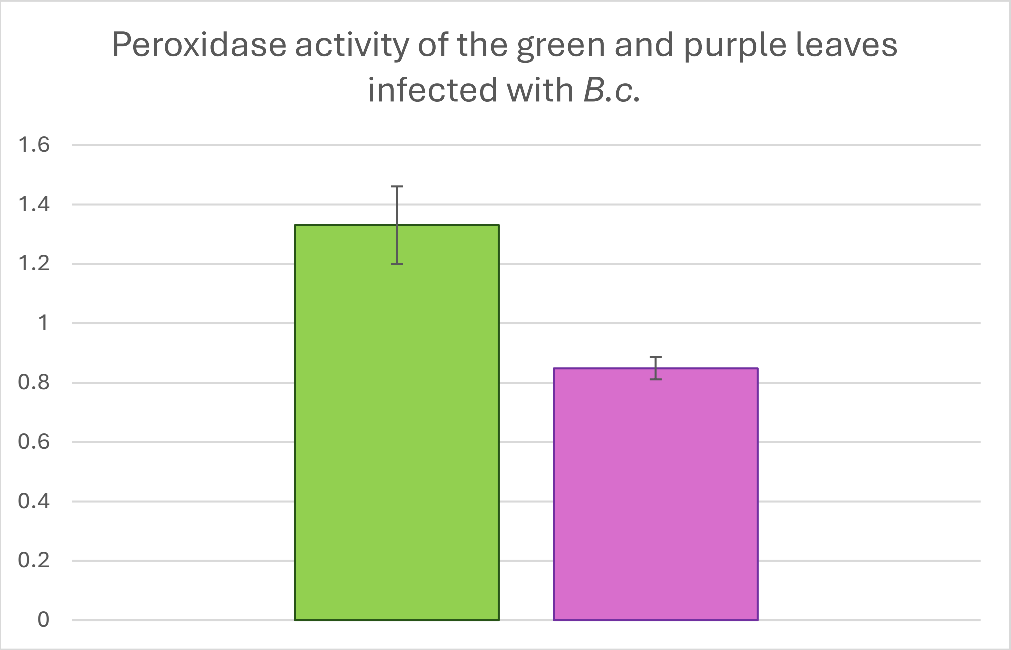
The fig 17. indicates that *Botrytis cinerea* infection is higher in green leaves than in purple leaves. Which indicates that's due to higher anthocyanin content, which acts like antioxidant and antifungal protection, the infection is reduced. Similar results were shown by (Bassolino et al., 2013) where anthocyanin rich areas of tomato showed lesser infection than the less pigmented regions.

Figure 18. – Superoxide dismutase activity of the green and purple leaves after 4 days of incubation (Source: Own work)



The enzymatic activity was also measured of the detached leaves (fig 18 & 19.). Superoxide dismutase (SOD) activity was higher in green leaves (375,17 U/g fresh weight) compared to purple leaves (119,11 U/g fresh weight). This difference suggests that anthocyanin-rich tissues are exposed to lower oxidative pressure due to the antioxidant role of anthocyanins. The enzymatic defence system, including SOD, is less induced in these tissues. In contrast, green leaves, lacking anthocyanin pigments, rely more on enzymatic detoxification of superoxide radicals. Similar patterns were reported by (Gould, 2004) and (Zhao et al., 2022) supporting the complementary relationship between anthocyanins and enzymatic antioxidants.

Figure 19. – Peroxide dismutase activity of the green and purple leaves after 4 days of incubation



Peroxide dismutase activity was higher in green leaves (1.3309 U/g fresh weight) compared to the purple leaves (0.8486 U/g fresh weight) as shown in the fig 19. This suggests that purple leaves have anthocyanins which acts as antioxidants, which can reduce the oxidative stress and therefore the plant require less peroxidase activity. However, in green leaves due to lower levels of anthocyanins and antioxidants, the depend on more on peroxidase to prevent oxidative damage and this leads to higher oxidative stress. Similar results were observed by (Wang et al., 2017), that the expression levels of POD in purple leaves are recorded lower than in the green leaves.

This similar trend supporting the theory that anthocyanin can reduce the need for enzymatic detoxification.

4. CONCLUSION

The current study shows that light exposure regulates anthocyanin production, antioxidant capability, and DNA methylation patterns in *Capsicum annuum* leaves. Analytical results found that leaves exposed to light had considerably greater total polyphenolic content (TPC), total flavonoid content (TFC), ferric-reducing antioxidant power (FRAP), and total monomer anthocyanin (TMA) than those that were covered and kept dark. These results prove that light functions as a positive regulator of secondary metabolite synthesis, therefore strengthening the plant's antioxidant defence mechanism.

Methylation-sensitive amplification polymorphism (MSAP) study confirmed the above results, indicating that light conditions influenced DNA methylation patterns. Many loci became more methylated in the dark, indicating that light exposure preserves a more open chromatin state favourable for anthocyanin biosynthesis gene expression, whereas darkness promotes methylation-linked gene silence.

The detached leaf tests revealed that purple leaves with greater anthocyanin content were less sensitive to Botrytis cinerea infection than green leaves. NBT and DAB staining indicated decreased amounts of reactive oxygen species (superoxide and hydrogen peroxide) in purple leaves, demonstrating anthocyanins' protective effect against oxidative stress and pathogen infection.

Overall, what we've discovered shows that light boosts anthocyanin accumulation and antioxidant capability while simultaneously influencing epigenetic control via DNA methylation. The presence of anthocyanins increases resistance to fungal infections, underscoring their dual role in plant defence as pigments and bioactive chemicals. These findings shed light on the relationship between environmental cues, secondary metabolism, and epigenetic regulation in *Capsicum annuum*.

5. SUMMARY

This study examined the impact of light exposure on anthocyanin biosynthesis, antioxidant capacity, and epigenetic regulation in *Capsicum annuum* leaves. Leaves were covered with aluminium foil to generate paired light-exposed and dark-treated samples. Total polyphenolic content, total flavonoid content, and ferric-reducing antioxidant power were measured using spectrophotometric assays.

Light-exposed leaves contained higher levels of phenolic compounds and showed greater antioxidant potential than shaded leaves. These findings indicate that light enhances secondary metabolism by promoting the biosynthesis of antioxidant molecules, including anthocyanins.

Epigenetic regulation was evaluated using a methylation-sensitive amplification polymorphism (MSAP) assay on DNA from the same leaf pairs. Darkness increased methylation at several loci, while light exposure reduced methylation. These results suggest that light maintains open chromatin to support anthocyanin biosynthesis gene expression, whereas darkness promotes gene silencing through methylation.

The physiological role of anthocyanin accumulation was assessed using detached leaf assays. Purple and green leaves were inoculated with Botrytis cinerea, and disease progression was monitored. Histochemical staining revealed significantly lower superoxide and hydrogen peroxide levels in purple tissues, indicating that anthocyanins reduce oxidative stress and enhance resistance to fungal infection.

In summary, light exposure increases anthocyanin accumulation, enhances antioxidant capacity, and alters DNA methylation patterns. Anthocyanins offer antioxidative protection and improve tolerance to Botrytis cinerea, underscoring their dual function as pigments and defence compounds. This study links environmental signals, secondary metabolism, and epigenetic regulation in *Capsicum annuum* leaves.

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8. LIST OF TABLES AND FIGURES

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: Dalli Sruthi Laya
Student's Neptun code: W3NP7P
Title of thesis: The effect of lightning on anthocyanin biosynthesis of Capsicum annuum
Year of publication: 2025
Name of the consultant's institute: Institute of Genetics and Biotechnology
Name of consultant's depament: 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

- not confidential thesis after the defence
- confidential thesis 5 years after the submission

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

Date: 2025 year November month 03 day

Student's signature

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[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

Dalli Sruthi Laya (name) (student Neptun code: W3NP7P)
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: 2025 year November month 03 day

Kovács Zsó
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:	Dalli Sruthi Laya
Neptun ID:	W3NP7P
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 lightning on anthocyanin biosynthesis of 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)
Brainstorming, language proofreading	Chat GPT 5.0	Literature

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 advisor I have no tools provided
by the university with which I can 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. November month 03 day

Kovács Zsolt

Signature of the Student **Signature of the Advisor/Supervisor**