THESIS WORK

Beatrice Konadu Agyapomaa
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Evaluation of secondary cell wall constituents and *in vitro* analysis of mutant *Capsicum annuum* plants

Primary Thesis Advisor:	**Pápai Bánk**
	PhD Student
Primary Thesis Advisor:	**Dr. Veres Anikó**
	Associate professor
Author:	**Beatrice Konadu Agyapoma YFIHGO**
	Full time
Institute:	**Institute of Genetics and Biotechnology,**
	Department of Genetics and Genomics

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Capsicum annuum is a world-wide known and consumed plant. Hungarian bell pepper is one of the most consumed vegetables in Hungary, so it is crucial to provide more fruits. Even though it originated from Central and South America (Aguilar Meléndez *et al.* 2009) it has an important role in Hungarian traditional cuisine and food consumption (Csóka *et al.* 2013). Sharma and Gupta (2017) reviewed the morphology and anatomy of pepper fruit, with a focus on *Capsicum annuum* L. They described the various morphological features of the pepper fruit, such as its shape, size, color, and texture. They also discussed the anatomical structures of the fruit, including the pericarp, placenta, and seeds. The authors highlighted the importance of understanding the morphology and anatomy of pepper fruit for breeding and genetic improvement programs.

Horticulture is a dynamic and an always-changing and constantly evolving sector which needs new ideas, plant materials, and cultivating methods to produce more considering the climate-changes and the always increasing population. The application of mutant traits to breeding lines has become extremely important since mutant plants may present some unique properties which may be useful in vegetable production. Laying, procumbent, decumbent phenotypes are commonly used as ornamental plant phenotypes but including them in greenhouse cultivation may open us new possibilities. Csilléry Gábor owns the biggest *Capsicum annuum* mutant collection in Hungary which contains the so called *pcx* and *tti* breeding lines. *pcx* plants exhibit a laying, vine-like growth habit and *tti* plants have slender, elongated spiraling stems. There are many proposed theories behind plant growth habit. The abnormal stem growth of these

mutants might occur due to lignin deficiency or an error in sensing the light or the gravity sensing process. Because of the economical relevance of the Hungarian bell pepper (Terbe *et al.* 2004), it is necessary to create and improve new cultivating styles to produce more. *Capsicum annuum* is not just important in food consumption, it also has a lot of vitamins, phytochemical compounds such as flavonoids, carotenoids and capsaicin which are important in the prevention of health issues (El-Ghorab *et al.* 2013). Understanding the background of this mutant phenotype might help in developing different cultivation techniques and aid its participation in pepper breeding programs.

OBJECTIVES

Main Objective:

The main goal of the experiments was to evaluate the response of the plants to environmental stimuli and their lignin content (secondary cell wall constituents).

Specific Objectives:

a. To check the response of the plants to light and gravity stimuli.

b. The time taken for the plants to adapt and react to a new gravitational vector.

c. Characterize them based on their reaction to gravity and light.

d. To check if there are any differences in lignin content between the mutants and the control plants.

2. LITERATURE REVIEW

2.1 *Capsicum annuum* taxology, morphology

Capsicum annuum L. is a dicotyledonous flowering plant which is grown worldwide. It belongs to the Solanales order, Solanaceae family and the *Capsicum* genus. *Capsicum* is an economically essential genus and contains at least 32 species native to tropical America. The mostly cultivated and consumed are the 5 domesticated *Capsicum* plants, which are *Capsicum annuum*, *Capsicum frutescens*, *Capsicum chinense*, *Capsicum baccatum* and *Capsicum pubescens* (Hernández-Pérez *et al.* 2020). The most widely accepted classification system is based on the work of Eshbaugh (1980), which divides the genus Capsicum into the five major species groups. The annuum species group includes the most widely cultivated pepper species, including bell peppers, jalapenos, and cayenne peppers. These peppers are characterized by their small flowers and round or oblong fruits. The baccatum species group includes peppers such as the aji and rocoto, which have large, pendant fruits and flowers with greenish-yellow petals. The chinense species group includes some of the hottest peppers in the world, such as the habanero and scotch bonnet, and is characterized by its small, white flowers and wrinkled fruits. The frutescens species group includes peppers such as the tabasco pepper, which is often used in hot sauce production, and is characterized by its small, white flowers and small, pointed fruits. The pubescens species group includes the rocoto and manzano peppers, which are characterized by their hairy leaves and large, round fruits. Within each species group, there is significant morphological variation, and many different cultivars and varieties have been developed. In addition to the traditional classification based on morphology, molecular methods have been used to study the taxonomy of pepper. The morphological and anatomical changes that occur during fruit development of chili pepper (*Capsicum annuum* L.) were studied by Ocampo et al. (2003). They found that the fruit of chili pepper undergoes significant changes in size, shape, and color as it develops. The fruit grows from a small green structure to a larger, red, mature structure. The morphology of pepper fruits is characterized by their shape, size, color, and texture. The pepper fruit is a berry that develops from the ovary of a flower, and its morphology changes as it matures. The study also revealed changes in the anatomy of the fruit, including the development of the placenta and the arrangement of the seeds.

The *Capsicum annuum* stem is branched densely about 60 cm in length. The flowers, which can be an off-white, or purplish color has nectars at the end of the corolla that helps to attract pollinator. They are generally self-pollinating plants, but with the help of insects ripening speed has increased in addition to ensuring symmetrical development of fruits. The fruits may be green, orange, white, yellow, or red when ripe (Arimboor *et al.* 2015). There are some new cultivars as well which have purple-colored fruits (Meng *et al.* 2022). The shape of the fruits has a wide spectrum of different forms (Cselőtei *et al.* 1993). The value of the pepper fruit is determined by the size, the color, the shape, and the taste all together (Paran *et al.* 2011).

2.2 Nutritional Value and Benefits

Capsicum annuum L., sometimes known as the sweet bell pepper, is a crucial food that humans consume on a regular basis. Vitamins C and E, provitamin A, and carotenoids are all present in peppers in good amounts (Materska&Perucka 2005). According to several studies by Amakura et al, 2002; Delgado-Vargas and Paredes-Lopez 2003; Materska&Perucka 2005, peppers also contain a variety of phenolics and flavonoids. Consuming peppers may prevent a number of diseases linked to free radical oxidation, including cardiovascular disease, cancer, and neurological disorders (Doll 1990; Hollman&Katan 1999; Harborne&Williams 2000; Delgado-Vargas&Paredes-Lopez 2003; Shetty 2004). These compounds are antioxidants that can lessen harmful oxidation reactions in human body. According to the USDA National Nutrient Database, Sweet pepper (*Capsicum annuum*), red, raw, nutrient value per 100g contains in (Table 1)

Table 1: *Capsicum annuum* contents and nutrient value per 100g

Contents	**Ratio**
Water	92.2g
Energy	26kCal
Energy	111kJ
Protein	0.99g
Total lipid(fat)	0.3g
Ash	0.47g
Carbohydrates	6.03g

Fiber	2.1g
Glucose	1.94g
Fructose	2.26g
Calcium	7mg
Iron,Fe	0.43
Magnesium,Mg	12mg
Phosphorus,P	26mg
Potassium,K	211mg
Sodium,Na	4mg
Zinc,Zn	0.25mg
Manganese	0.122mg
Vitamin C	128mg
Thiamin	0.054mg
Riboflavin	0.085
Niacin	0.979mg
Pantothenic acid	0.317mg
Vitamin B-6	0.291mg
Folate	46µg
Vitamin A(RAE)	157µg
Carotene(β)	1620µg
Carotene(α)	20µg
Cryptoxanthin	490µg
Lutein+Zeanthin	51µg
Vitamin E	1.58mg
Vitamin K	4.9µg

According to Frank et al,2010 bell peppers come in a variety of colors, but green bell peppers are the most popular and are consumed the most. The main element influencing consumer purchase decisions is the hue of sweet bell peppers. In addition to hue, different peppers of different colors

have reported varying nutrient contents, such as vitamin C concentration (Simonne et al, 1997; Frank et al,2001).

Vegetables include essential pigments called carotenoids and flavonoids, which frequently give them an orange or red hue (Delgado-Vargas&Paredes-Lopez 2003). Capsanthin and carotene are two carotenoids found in peppers (Howard, 2001). Chlorophyll and the chloroplast-specific carotenoids are responsible for the green color of peppers (Marin et al, 2004).

Peppers' yellow-orange hue is created by beta- and gamma-carotene, zeaxanthin, lutein, and beta-cryptoxanthin (Howard,2001). The carotenoid pigments capsanthin, capsorubin, and capsanthin 5,6-epoxide are what give peppers their red color. The various levels of those chemicals may account for the peppers' various colors. The varied colors of bell peppers may have variable antioxidant activity because those chemicals have an antioxidant role.

2.3 *Capsicum annuum* L. Production in Hungary

Pepper (*Capsicum annuum* L.) is an important crop in Hungary, both for domestic consumption and export. Hungary is one of the major producers of pepper in Europe, and its climate and soil conditions are ideal for pepper cultivation (Helyes et al., 2017).

Pepper has been cultivated in Hungary for centuries, and it has played an important role in Hungarian cuisine. Hungarian paprika, made from dried and ground red peppers, is a key ingredient in many Hungarian dishes, such as goulash and paprikash. The first paprika factory in Hungary was established in 1859, and by the late 19th century, paprika had become a major export crop (Kovács, 2005).

During the communist era, pepper production in Hungary was heavily subsidized by the government, and farmers were required to sell their crops to state-run cooperatives at fixed prices. After the collapse of communism in 1989, Hungary underwent a transition to a market-based economy, and the pepper industry underwent significant changes. Many small-scale farmers went out of business, and larger farms consolidated and modernized their production methods (Helyes et al., 2017).

2.3.1 Current State of the Industry

Today, Hungary is one of the largest producers of pepper in Europe, with an annual production of around 40,000 tons (FAOSTAT, 2021). Most of the Hungarian pepper production is focused on

sweet paprika, which accounts for around 80% of the total production (Helyes et al., 2017). The main production regions are in southern Hungary, particularly in the counties of Szeged and Békés.

Hungarian pepper production is characterized by a mix of large-scale commercial farming and small-scale family farming. The larger farms use modern technologies such as drip irrigation and plastic mulching to increase yields and improve efficiency, while the smaller farms rely more on traditional methods (Helyes et al., 2017). The industry is dominated by a few large companies, such as Univer Product Zrt and Fűszerpaprika Zrt, which together account for around 70% of the market share (Helyes et al., 2017).

2.3.2 Challenges and Opportunities

Hungarian pepper farmers face several challenges, including competition from other pepper-producing countries, fluctuating prices, and climate change. Spain and Turkey are the largest competitors for Hungarian sweet paprika, and their lower labor costs and higher yields make it difficult for Hungarian farmers to compete on price (Helyes et al., 2017). Climate change is also a concern, as it can lead to extreme weather events such as droughts and floods, which can damage crops and reduce yields (Várallyay, 2020). However, there are also opportunities for the Hungarian pepper industry. The growing demand for organic and sustainable products presents an opportunity for Hungarian farmers to differentiate themselves from competitors (Helyes et al., 2017). In addition, there is potential for increased exports to non-EU countries such as the United States and China, where demand for paprika and other spices is growing (Helyes et al., 2012).

2.4 Pepper Breeding

Breeding of pepper is an important agricultural activity that aims to improve the yield, quality, and disease resistance of the crop. The aims of pepper breeding are manifold, but they generally focus on improving the crop's resistance to biotic and abiotic stressors, as well as enhancing its nutritional value and market appeal. Modern plant breeders can adapt mutant traits into cultivars to improve them creating new cultivating styles (Shalaby *et al.* 2013). In Hungary breeders mostly use conventional breeding methods. In case of *Capsicum annuum* the methods are mass selection, pure line selection, pedigree method, single seed descent, backcross method, heterosis

breeding and rarely induced mutagenesis (Kuhn *et al.* 2016). The goals of conventional pepper breeding are to produce cultivars which possess resistance against biotic and abiotic stress factors while they produce high quality fruits (Devi *et al.* 2021). Normally the breeding processes take more years, but we can shorten that time if it is supported by biotechnological tools (Barroso *et al.* 2019).

According to the Food and Agriculture Organization of the United Nations (FAO), the key objectives of pepper breeding include:

1. Developing varieties that are resistant to pests and diseases, such as bacterial wilt, anthracnose, and viral diseases.

2. Improving yield potential and crop uniformity to ensure consistent production.

3.Enhancing the nutritional value of the crop, including its vitamin C content.

4. Developing varieties with desirable culinary characteristics, such as flavor, aroma, and heat level.

To achieve these objectives, breeders use a variety of methods, including traditional and modern techniques. Traditional breeding methods involve the selection and hybridization of plants with desirable traits, while modern techniques, such as molecular breeding and genetic engineering, allow breeders to manipulate the plant's DNA more precisely. One traditional breeding technique used in pepper breeding is mass selection, which involves selecting the best-performing plants based on a particular trait and breeding them to produce the next generation of plants. Another technique is backcrossing, which involves crossing a plant with desirable traits with a parent plant to produce offspring that have the desired traits but retain the genetic makeup of the parent plant. Modern breeding techniques, such as marker-assisted breeding and genetic engineering, allow breeders to manipulate the plant’s DNA more precisely. Marker-assisted breeding involves identifying specific genes associated with desirable traits and using molecular markers to track them in breeding populations. Genetic engineering involves introducing specific genes into the plant's genome to confer desirable traits, such as disease resistance or improved nutritional value. Several studies have been conducted on the breeding of pepper, demonstrating the effectiveness of different breeding techniques. For example, Zhang et al. (2017) used marker-assisted selection to develop a new pepper variety with improved resistance to bacterial wilt. In another study,

García-Salas et al. (2017) used traditional breeding methods to develop a new pepper variety with enhanced antioxidant activity and higher levels of vitamin C.

2.5 Molecular methods in breeding

Plant breeding in a broad sense can be defined as the science, art and business of improving plant species for the benefits of man. It began first with plant domestication where several species of plant were brought under management and cultivation by man (Xu, 2010). In recent times like these, the focus on breeding in plants has shift towards the use of modern tools for crop acceleration and improvement. More emphasis is placed on molecular methods used for breeding plant species as compared to conventional breeding methods.

Molecular methods used in breeding programs include; genetic engineering, molecular marker-assisted selection, genomic selection, gene transfer/GMO, genome editing, and mutation breeding. These methods make use of gene manipulation performed at DNA molecular levels to improve and develop plant cultivars for the desired purpose (Yang *et al.* 2015). Research study reveals that genome editing which is considered as one of the molecular methods in plant breeding has played a significant role in crop improvement. It involves the insertion, deletion, or substitution of a foreign gene of one organism into another organism's DNA. The new transformed sequence is incorporated into the genome of the host organism to replicate, multiply, and then give rise to new recombinants species that are variant in forms and structures (Ahmar *et al.* 2020).

Molecular markers are reported to have been used in plant breeding activities in most agricultural crops to achieve success. They include technologies like restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSRs), single-nucleotide polymorphism (SNP), and diversity arrays technology (DArT) markers. These molecular markers are categorized into groups on the basis of their mode of action, mode of detection and mode of transmission (Nadeem *et al.* 2018). Also, Marker Assisted Selection which is reported to be highly effective for simple traits controlled by many genes has been useful in plant breeding programs. It allows plant breeders to partially remove phenotyping and conduct selection experiment at an early stage with high level of selection precision (McGowan *et al.* 2021). A similar study conducted by other researchers posits that molecular

markers are accurate in terms of amplification of genetic material of crop species *in vitro* using the polymerase chain reaction technique. This technique gives room for the diagnosis of polymorphism in DNA sequences that are amplified at various weights density depending on the kind of marker employed in the study (Al-Hadeithi & Jasim 2021).

Plant genomic selection is considered as a crucial technology that could also be used to enhance sustainable agriculture in the world. That is, the crop genome sequences provide the basis for the identification of agronomically relevant variation in crop species. Genomics accelerating plant breeding help to leverage genetic components of agronomic traits by way of increasing the yield component of crops, reducing environmental challenges as well as climate change impacts on agriculture (Hu *et al.* 2018).

Mutation breeding is essential for the development of new varieties of crops for sustainable production. Mutation can be spontaneous or non-spontaneous. It is regarded as one of the principal factors behind evolution of crop species that can create new alleles responsible for genetic diversity. Report reveals that spontaneous mutation is very slow and thus, induced mutation can be used to facilitate the rate of genetic diversity of crops species so that plant breeders can successfully exploit the different varieties in plant breeding programs (Chaudary *et al.* 2019). Mutation breeding uses mutagenic agents such as radiation and some forms of chemicals that could cause mutation to take place leading to the creation of genetic variations from which superior and desired mutants of crop species can be selected. Radiation mutation together with tissue culture has played a significant role in plant breeding by way of introducing new techniques that have helped to accelerate breeding time (Novak F.J. 1992).

2.6 Biosynthesis of Secondary Cell Wall and Lignin

Pepper is an important spice crop that is grown in various parts of the world. The secondary cell wall and lignin biosynthesis pathway is important in the development and growth of the pepper. The secondary cell wall is a complex structure that provides structural support and protection to the plant. Lignin is a major component of the secondary cell wall, providing rigidity and strength to the cell wall. The biosynthesis of the secondary cell wall and lignin occurs through a complex pathway involving various enzymes and regulatory factors in the plant. One of the key enzymes involved in the biosynthesis of lignin is phenylalanine ammonia-lyase (PAL), which converts phenylalanine into cinnamic acid. Cinnamic acid is then converted into various monolignols,

which are the building blocks of lignin. The monolignols are polymerized into lignin through the action of various enzymes, including peroxidases and laccases. The regulation of lignin biosynthesis is complex, involving the coordinated action of various transcription factors and signaling pathways. For example, the MYB transcription factors are known to regulate lignin biosynthesis in pepper, and their overexpression has been shown to increase lignin content in pepper plants. The biosynthesis of the secondary cell wall and lignin in pepper has important implications for plant growth and development. Lignin content and composition affect the mechanical strength of the stem, influencing the plant's ability to withstand biotic and abiotic stresses. Additionally, lignin content and composition affect the digestibility and nutritional quality of the plant, making it an important factor in animal feed and biofuel production. Several studies have been conducted on the biosynthesis of secondary cell wall and lignin in pepper, providing valuable insights into the regulation and function of this pathway. For example, Chen et al. (2020) used transcriptomic analysis to identify key genes involved in lignin biosynthesis in pepper, while Kim et al. (2021) investigated the function of a MYB transcription factor in regulating lignin biosynthesis in pepper.

2.7 Mutations related to lignin deficient plants

Lignin is reported to be a highly complex phenolic polymer which provides mechanical strength, rigidity, and resistance of microbial infections to the cell walls of plant (Rogers & Campbell, 2004). The lignin polymer in plants' cell walls is formed by oxidation process where oxidative coupling of hydroxycinnamyl alcohol monomers, or monolignols are derived from the phenylpropanoid pathway (Panda *et al.* 2020). It provides hydrophobic surface which is important for transportation of water, and provides a barrier against pathogens attack (Mackay *et al.* 1997). Naturally, some plant species lack lignin in that they do not have the conventional vascular tissues which contain the substance lignin. An example of such plant species is the Bryophytes. Bryophytes are deficient in lignin due to the absence of conventional vascular tissues. Further study has shown that lignin provides recalcitrant structure that embeds cellulose together with hemicellulose. The recalcitrant structure of lignin is a major limitation of utilizing the nutritional polysaccharides for animal feed stock and production of bioproducts (Christensen & Rasmussen, 2019). Similar study suggest that lignin contributes to the

recalcitrance of biomass composition in plant. To overcome this problem, modification of monolignol and incorporation of monomers have been proven to be effective methods for reducing biomass recalcitrance in plant species (Muro-Villanueva *et al.* 2022).

Since time immemorial, the focus on altering the lignin of plant cell walls has been on targeting individual steps which are involved in lignin biosynthesis. For instance, transgenic tobacco plants which have a downregulated cinnamol-CoA reductase activity have exhibited some alterations in development. Examples of such alterations are; a reduced size and a collapsed vessels of tobacco plants (Jones *et al.* 2001). Some lignin mutants by mutagenesis have been described to be useful in the model plant *Arabidopsis thaliana*. That is, the lignin mutants have helped to overcome the growth deficiency of the plants by mutating the mediator5a and mediator5b subunits in the *c3′h* background (Smith *et al.* 2022). Another research study has proposed Sekizaisou to be a newly natural mutant that is deficient in cinnamyl-alcohol dehydrogenase. This mutant helps to catalyze the reduction of hydroxycinnmaldehydes in the monolignol biosynthesis pathway of plant species (Science, 2021). Again, Lignin mutants have been reported to be useful in herbaceous plants. The Brown midrib (*bm*) mutants that are found in monocots have a decreased in lignin content and a modified lignin composition (Mackay *et al.* 1997). In maize plant, the brown midrib (bm) mutants exhibit a reddish-brown pigmentation of the leaf midrib and stalk pith. This has an association with lignified tissues after the plants have had about five expanded leaves. The mutations of these mutants are as a results of identical factors and that the “bm character” segregated as “a simple mendelian recessive” trait (Barrière *et al.* 2013).

2.8 Response of plants to different environmental factors

2.8.1. Gravitropism and Phototropism

According to Hopkins & John 2011, plants respond to stimulating signals known as tropisms in their environment. These external stimulating signals are responses to light (phototropism), gravity (gravitropism) and touch (thigmotropism). Roots have been confirmed to grow positively to gravity whilst the shoot system grows negatively towards the gravity vector (Korell & Kiss 2002). Also, shoots have been confirmed to have a positive phototropism towards

blue light whilst roots display negative one (Okada & Shimura 1992) but display a positive one towards red illumination (Ruppel *et al.* 2001).

Phytochromes are photoreceptors (they perceive red and far-red light, cryptochromes and phototropins) which are present in plants. It has been suggested that plant organs such as the roots and shoots have these phytochrome responding signals (Jiao *et al.* 2005, Montgomery 2008). Again, these light signals are primarily received in the shoots and then transduced to other parts of the plants ie. roots (Hall et al, 2001). Tasaka et al, 1999 and Boonsirichai *et al.*, 2002 have posited that gravity's magnitude and direction believed to be almost constant makes gravitropism as an adjustment mechanism when the plants sense the alignment of organs in relation to the direction of gravity. He further added that because gravity acts on mass, some organisms rely on statoliths or otoliths (heavy cellular components) to sense the gravitational direction or vector.

Amyloplasts which are unique plastids can act as these statoliths for gravity sensing. They accumulate dense starch particles in statocytes (amyloplasts specific cells) and drop in the gravitational direction (Morita and Tasaka 2004). This sedimentation suggests that the starch statolith hypothesis within the statocytes is the gravity sensing trigger (Sack 1997; Morita and Tasaka,2004). Studies conducted by Blancaflor *et al.*, 1998 and Fukaki *et al.*, 1998 on molecular and genetic analysis of Arabidopsis thaliana showed that the endodermal cells and the root cap columella cells that contain sinking amyloplasts in their shoots and roots act as statocytes. There have been several studies by various studies to ascertain the starch statolith hypothesis in phosphoglucomutase(pgm) mutant. These studies reveal that these mutants show an abnormal starch making in their shoots and roots and thus the amyloplasts do not sediment in the gravity's direction of the mutant statocytes (Caspar and Pickard, 1989; Kiss *et al.*, 1989, 1997; Weise and Kiss, 1999). This same study further proves that there is a positive relationship between the reduction in gravitropism and the reduction in starch content, confirming that the amount of starch affects the magnitude of gravity response.

There are three procedures involved in gravity direction perception. These steps include: noticing the change in the gravity vector, transduction and the asymmetrical growth response. Sedimentation of the amyloplasts within the root cap columella cells and the layers of endodermal cells in the hypocotyls and stem (Kiss 2000; Sack 1991) marks the beginning of the first step. The next process is transduction, and this involves hormones (Kaufman et al, 1995;

Muday, 2001). After these steps, a curvature response is established that allows the organs to begin growth at a specified angle from the gravity vector (Figure 1.) (Masson *et al.*, 2002). Several nonhormonal regulatory messengers may also be involved in the gravitropic pathway. These include cytosolic Ca2þ ions (Plieth and Trewavas, 2002), inositol 1,4,5-trisphosphate (IP3) (Perera *et al.*, 1999), protein phosphorylation (Chang *et al.*, 2003; Rashotte *et al.*, 2001),phospholipase A2 (PLA2) (Lee *et al.*, 2003), the cytoskeleton network (Blancaflor, 2002; Friedman *et al.*, 2003b; Hou *et al.*, 2004), pH (Fasano *et al.*, 2001; Scott and Allen, 1999), reactive oxygen species (ROS) (Joo *et al.*, 2001), and nitric oxide (NO) (Hu *et al.*, 2005). Despite several evidence showing that the root caps are the site of gravity perception (Boonsirichai *et al.*, 2002), it still requires a means of communication between the sensing cells and those that respond in the elongation zone. This is different in coleoptiles, hypocotyls, epicotyls and infloresence stems as gravity can be perceived along the entire responding region (Fukaki *et al.*, 1998; Tasaka *et al.*, 1999). This further suggests that the spatial relationship between gravity sensing and responding tissues of the roots and shoots shows the existence of a mechanistic difference between shoots and roots in terms of signal transduction and transmission.

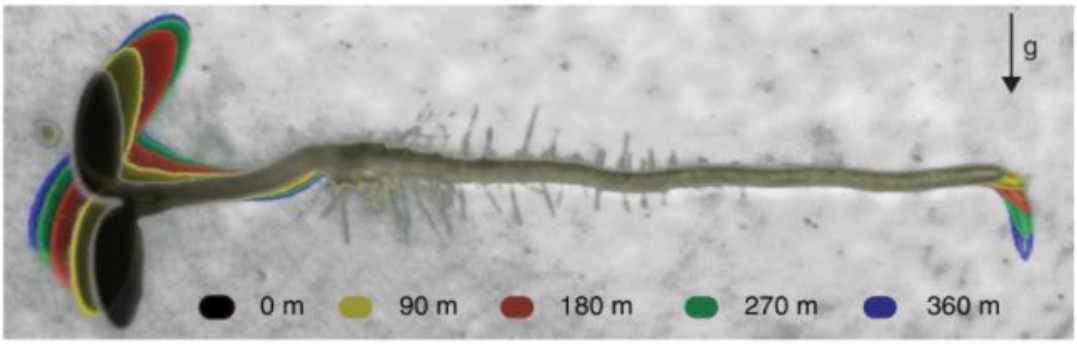


Figure 1: Arabidopsis seedling root and hypocotyl develop opposite curvature responses to gravistimulation (Su et al. 2017)

2.8.2. Phototropism

Plants are capable of adjusting their growth in response to their surrounding light environment. This phenomenon is known as phototropism (Holland *et al.*,2009). Plant stems exhibit positive phototropism whilst roots assume a negative phototropic state. This is particularly important for

germinating seeds that require sunlight for growth and developing roots especially under drought conditions (Galen *et al.*, 2004, 2007a,b). Lower plants such as ferns and mosses also rely on phototropism but use a different mechanism as compared to flowering plants (Grolig *et al.* 2000; Hartman *et al.* 1983; Nozue *et al.* 1998). Similar to gravitropism, phototropism exists in three distinct steps: light signal perception, transduction of signal and the growth response of the organs in line of the transduced signal. Studies conducted have shown several proteins involved in light sensing during flowering. Mutant plants with reduced phototropisms were used in these studies. According to Christie and Briggs (2001), the primary photoreceptors in angiosperms related to phototropism is the blue light photorecepetor which is also involved in the perception of UV-A and green wavelength.

3. MATERIALS AND METHODS

3.1. Plant materials

The mutant breeding lines used for this experiment was provided by Csilléry Gábor from the biggest pepper mutant collection in Hungary. The breeding lines involved are coded *pcx* (procumbent plant) and *tti* (tortuous internode).

The *pcx* mutant phenotype shows a procumbent stem growth habit, which means it has an abnormal growth habit. The first pcx phenotype was identified in the F3 progeny of a spice type bell pepper breeding line, whose fruits ripe from dark green color to red. They present various inconsistent growth habits. Since the stem structure is significantly rigid the laying phenotype is not caused by the lack of water transportation. The *pcx* pepper plants were given as seeds which makes the work more difficult. Despite knowing the plants are self-pollinated not all the progeny show the procumbent phenotype (Figure 2-3-4.).

The *tti* phenotype was identified in the F3 progeny of a Cecei type breeding line, that have fruits which ripen from white to red color. The *tti* plants have long hypocotyls and they grow a slightly spiraling slender stem. The inter-nodes of the plants have almost double the length compared to the average bell pepper inter-node length. This mutant trait is hard to observe in the very early age, the plants present the elongated phenotype only after growing 3-4 leaves (Figure 5).

As control, the well-known Hungarian cultivar ‘Fehérözön’ was used. The control pepper seeds were obtained from Royal Sluis Magrovet Kft., Kecskemét, Hungary.

Figure 2: pcx mutant pepper plants in a greenhouse in Szentes

Figure 3: pcx mutant pepper plants in a greenhouse in Szentes

Figure 4: Laying and non-laying *pcx* mutant pepper plants in the Department of Genetics and Genomics

Figure 5: *tti* mutant pepper plants in a greenhouse in Szentes

3.2. Preparation of MS media

For our experiments we used standard MS medium following the methodology of Murashige and Skoog, 1962. Table 2 contains all the ingredients of the standard MS medium.

Table 2: MS medium components

Content	**Ratio**
MACROELEMENTS	**mg/L**
KNO_3	1900
NH_4NO_3	1650
$CaCL_2x\ 2H_2O$	440
$MgSO_4$	370
KH_2PO_4	170
MESOELEMENTS	**mg/L**
$FeSO_{4\,x}7H_2O$	27.80
Na – EDTA	37.30
MICROELEMENTS	**mg/L**
$MnSO_4$ x $4H_2O$	22.30
$ZnSO_4$ x $7H_2O$	8.60
H_3BO_3	6.20
KJ	0.83
Ma_2MoO_4 x $2H_2O$	0.25
$CuSO_4$ x $5H_2O$	0.025
$CoCL_2$ x $6H_2O$	0.025
VITAMINS	**mg/L**
Inositol	100.00
Thiamine, B_1	0.10
Nicot.acid, B_3	0.50
Pyridoxine, B_6	0.10
Glycine	2.00
OTHERS	**g/L**
Plant Agar	8.00
Saccharose	30.00

***Ph -5.6-5.8**

Firstly, the macro, micro and meso elements were mixed together in a 500 ml flask. Then the solid components such as myo-inositol and sugar were added. Pour the contents of the flask into a volumetric flask and topped up with distilled water to 500mls. The contents were then put back into the flask and the Ph checked using a Ph meter ensuring that the Ph was on the optimal level between 5.6-5.8. The agar was then weighed and poured into the solution. It is then sealed completely using an aluminium foil and put into an autoclave for 20 minutes. The aim of this step is to sterilize the media. After sterilizing the media, it is cooled down by swirling in cold water. After the media cooled, it was taken to the laminar flow together with already sterilized jars and poured into them and after which it was covered tightly using an aluminium foil. Hormone supplementation was not added to this experiment.

3.3. Seed sterilization and *in vitro* conditions

3.3.1. Materials for sterilization

For preparing *in vitro* plant cultures the seeds of *pcx, tti* and 'Fehérözön' were used.
Materials for sterilization protocol are summarized on Table 3.

Table 3: Solutions and instruments for sterilization process

• 1% Hypochlorite solution
• 70% ethanol solution
• Sterile distilled water
• Sterile flasks

The quantity of seeds was sterilized based on the number of seeds to be used. For the evaluation of the plants' reaction to light and gravity 4 seeds were put in each jar. For evaluation of the amount of time needed for the plants to react to gravity only one seed was put into each glass vessel. For sterilizing the distilled water, an autoclave was used for 25 minutes once it began to boil. The sterilization of glassware was done using dry heat treatment of hot air sterilizer on 200° for 120 minutes.

3.3.2. Sterilization process

The following steps were carried out under completely sterile conditions using a laminar air flow chamber. Firstly, seeds were put into small pre-sterilized flask and 70% ethanol was poured on them. They were then swirled for 45 seconds and the alcohol was poured out. A 1% hypochlorite solution was put on them and the flask shaken for 20 minutes. After completing this step, the hypochlorite solution was poured out and the seeds were rinsed three times with sterile distilled water. The sterilized seeds were then cultured onto the MS plant media distancing them some few centimeters away from each other not to block each other during germinatio and growth. They were then covered tightly using a transparent foil ensuring that no pathogens should be able to get inside. The cultures were then incubated in a phytotron chamber.

3.3.1 *In vitro* phototropic and gravitropic experiments

The samples were then taken to the growth chamber. Seeds were germinated on 25±1°C with 16 h–8 h light-dark periods and 5000 lux light intensity.

In this experiment, the methodology mentioned by Grube *et al.* 2003 was followed (Figure 6). For the evaluation of the plants' reaction to light and gravity the first set of the seeds were covered in foil to restrict them from having access to light. The others that were not covered was sent normally to the growth chamber. In the growth chamber the plants have access to light beaming from only one side of the equipment. Further experiments were carried out two weeks after germination. The glass jars with the 2 weeks old plants were inclined at 90°. Documentations of plant response began after 24 hours.

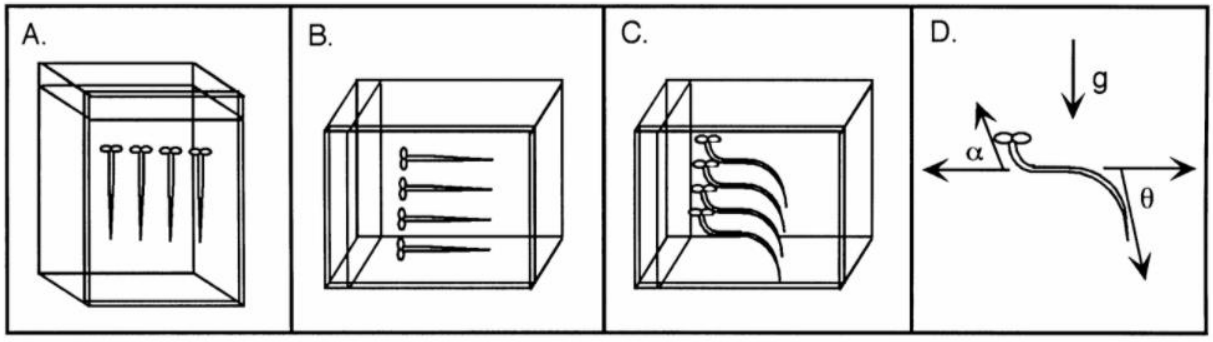


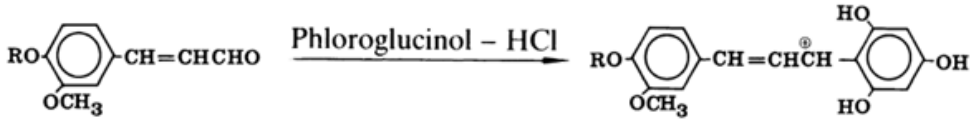
Figure 6: Evaluation of gravitropic response in in vitro plants (Grube *et al.* 2003)

For evaluation of the amount of time needed for the plants to react to gravity all the *in vitro* plants were grown without any coverage. For this experiment, the methods stipulated by Ajala *et al.* 2019 was used. Two weeks after germination, the glass vessels were inclined at 90°. The plants were documented in every hour for 8 hours. The gravitropical curvature of their stem was measured using ImageJ image analysing software and a graph plotted using MS Excel 365 to establish a comparison between the mutant *pcx* breeding line and the control cultivar.

3.5. Staining the lignin content of the stem

For checking the lignin content of the stems, the 'Color Reaction with Phloroglucinol-Hydrochloric Acid' method by Nakano *et al,* 1992 was used to check if there is any difference between the mutant breeding lines and the control. The reagent was prepared by mixing 50 ml of a 2% solution of phloroglucinol in 95% ethanol and 25 ml of concentrated hydrochloric acid. Since the reagent is not very stable, the phloroglucinol solution is used immediately after preparation. According to the author to store the phloroglucinol solution, one must put it in a dark glass and mix with the hydrochloric acid immediately before usage.

The Phloroglucinol-Hydrochloric test is sensitive to coniferaldehyde structures in lignin (Figure 7.) so it is applied dropwise on plant explants. For the experiment, stem parts from previously prepared 4 weeks old *in vitro* plants were used. According to the protocol the color reaction shows a reddish-violet color on lignified tissues. The color reaction was viewed and documented using stereo microscope on 16x magnification.



(1) R = H or alkyl

Figure 7: Phloroglucinol-Hydrochloric test on coniferaldehyde structures in lignin (Nakano&Meshitsuka 1992)

4. RESULTS AND DISCUSSION

4.1. Phototropic and gravitropic response of *pcx* plants

The aim of this experiment was to determine if the *pcx* mutant breeding line possess the ability to adapt different environmental stimuli such as light and gravity. The results can be seen on Figures 8-9-10.

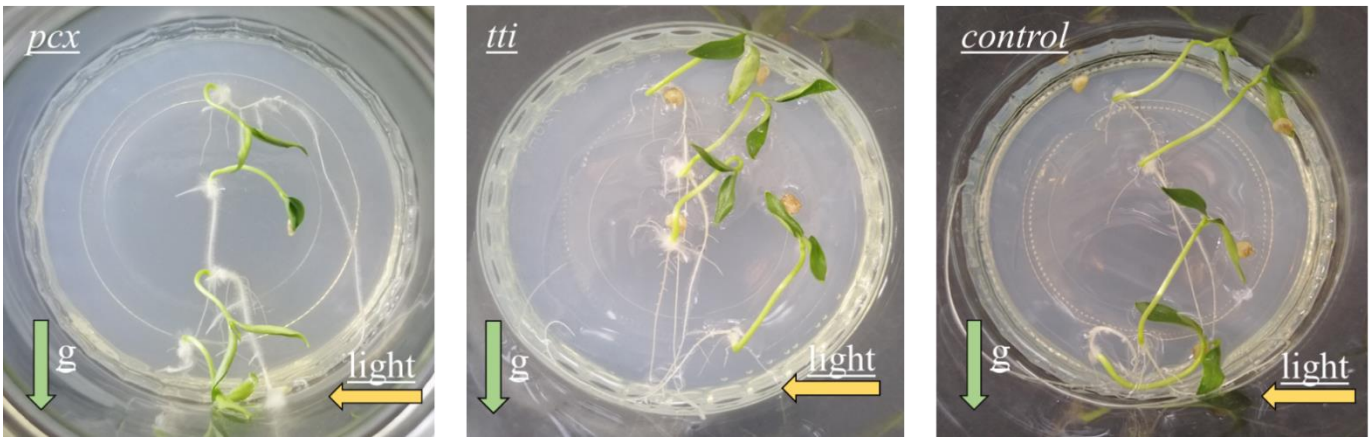


Figure 8-9-10: pcx, tti and control plants 24 hours after bending, **g** - direction of the gravity vector, **light** - direction of light beaming from only one side.

The *in vitro* germinated plantlets displayed that all the genotypes were able to sense and react to light following its direction. In all cases roots started to grow in the direction of the gravitational pull. The *pcx* stems did not show any sign of following the gravitational force while the control plants started bending upwards following the vector opposite to gravitational pull.

Figure 11-12-13: *pcx*, *tti* and control plants germinated in completely dark environment, 24 hours after bending, **g** - direction of the gravity vector.

As results of this experiment, it was identified that even in completely dark environment all the control plants were able to sense the gravitational force and grow their hypocotyls in the opposite direction. The *pcx* plants had their hypocotyls growing in random directions. The *tti* plants reacted and sensed the gravity but the stem structure was too weak to keep the stem upwards. In the absence of light therefore it can be suggested that the mutation affecting the *pcx* plants' stem growth is not influenced by the process of gravitropism or, in other words termed as anti-gravitropic plants.

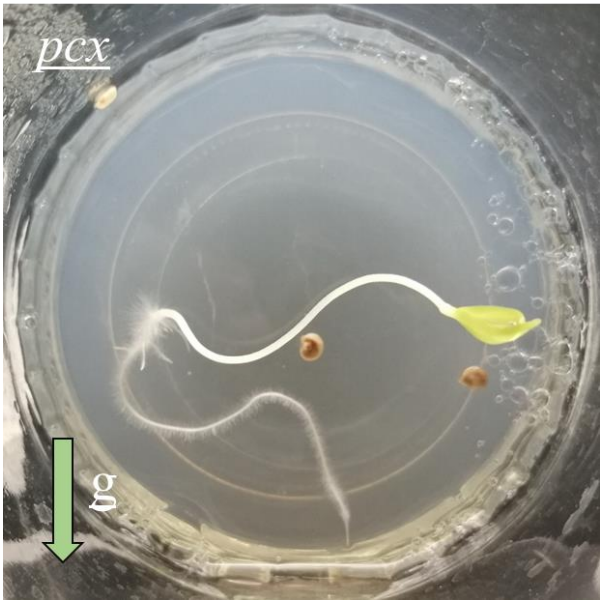


Figure 14: *pcx* plant germinated in completely dark environment, 24 hours after bending, g direction of the gravity vector.

In dark environment in all cases the roots started to follow the gravitational vector and started to grow downwards.

4.2. Time course of gravitropic curvature of *pcx* and control plants

The main purpose of this subsequent experiment was not just to determine if the *pcx* mutant breeding line is able to react to gravitational pull but to check the time amount they need to follow the gravitational pull since the growth randomization was more observed in this line. It was worth interesting to know if their ability to follow the gravitational vector only occurs later or not at all during the experiments. Since only the *pcx* plants did not show any immediate reaction towards the gravitational force further experiments were performed on them.

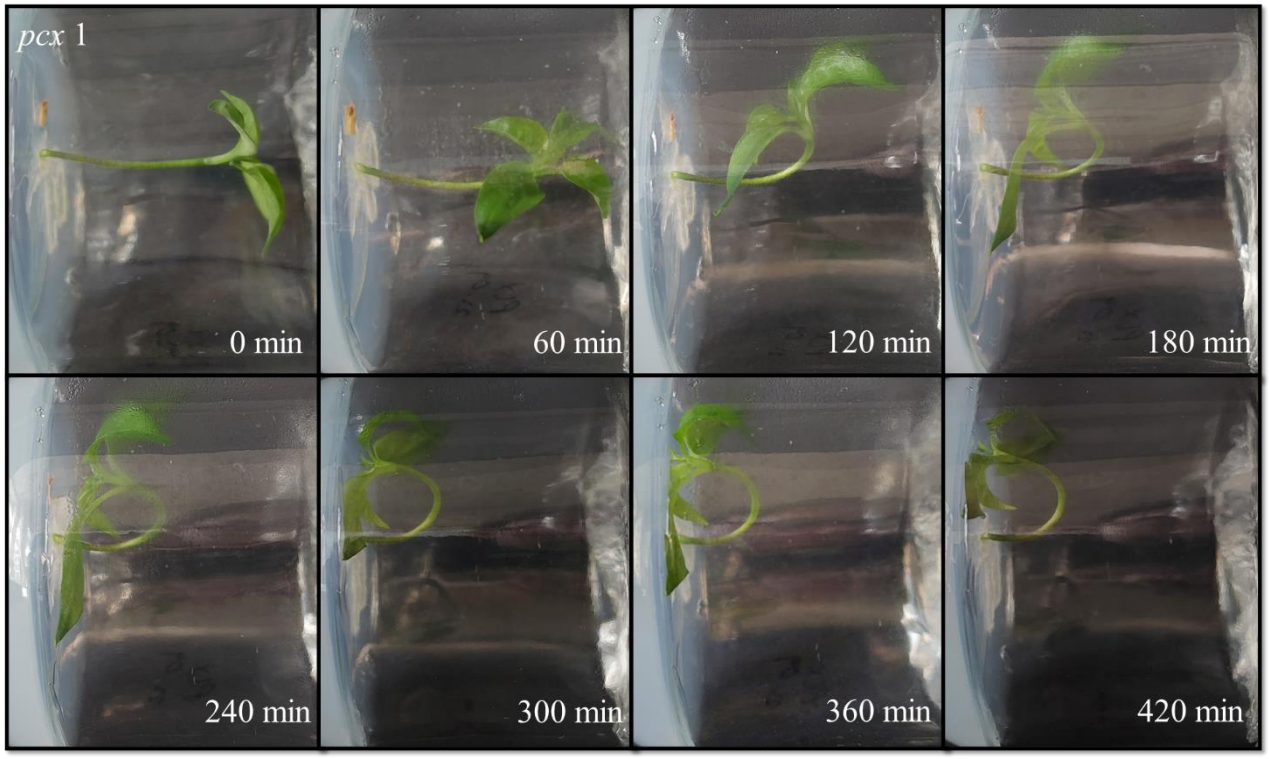


Figure 15: Time course of upward graviresponse of hypocotyls of *pcx* plant

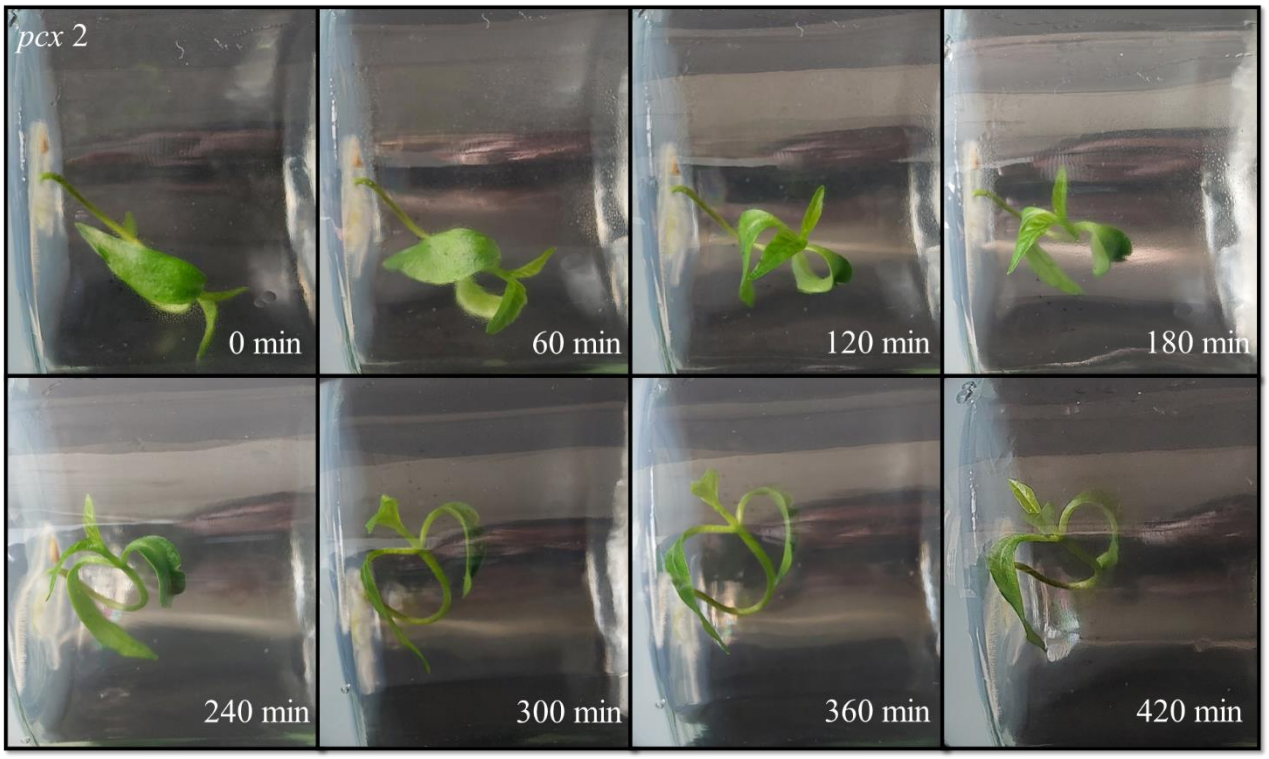


Figure 16: Time course of upward graviresponse of hypocotyls of *pcx* plant

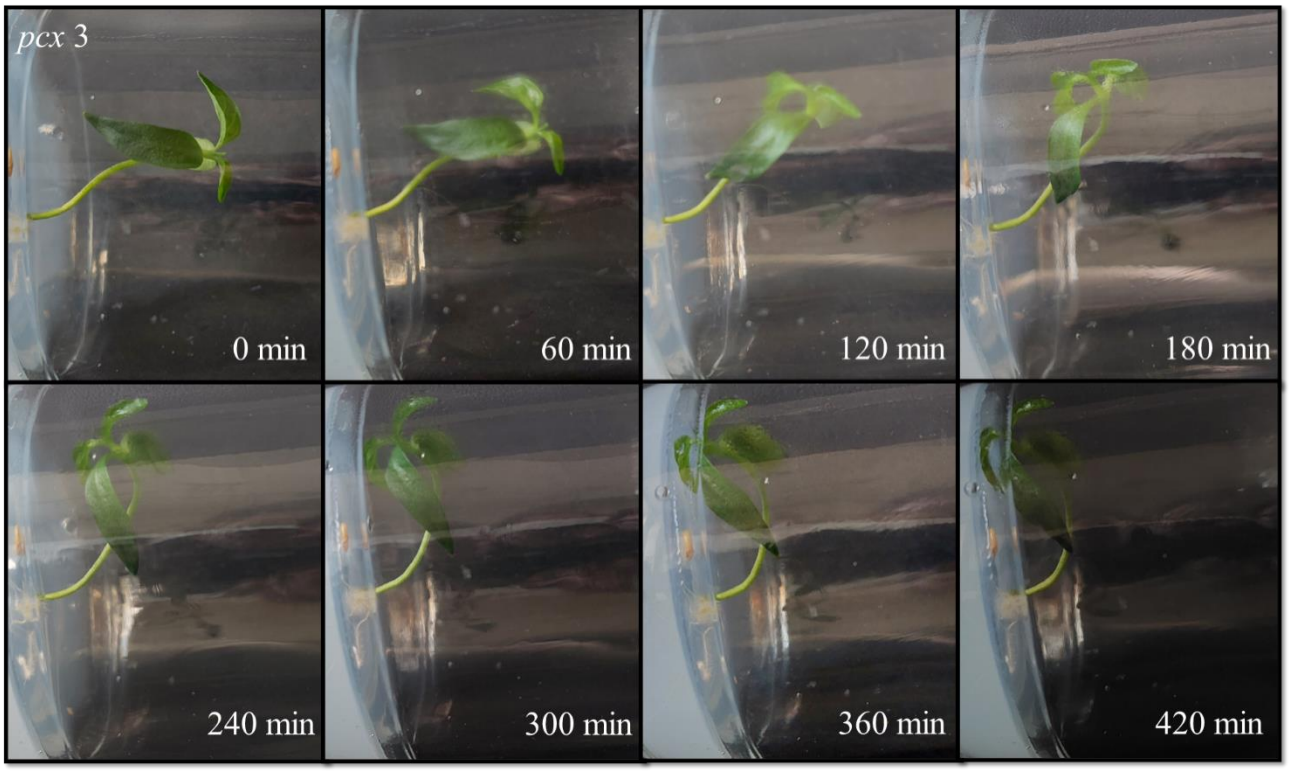


Figure 17: Time course of upward graviresponse of hypocotyls of *pcx* plant

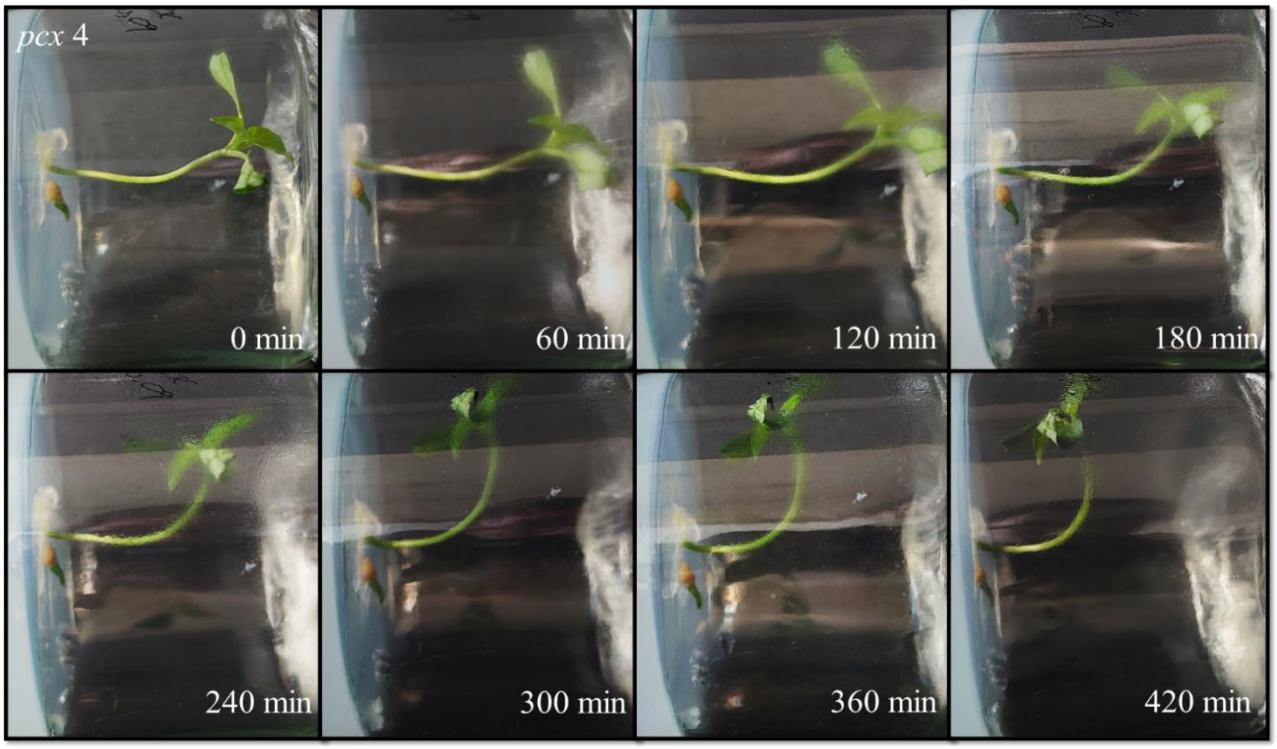


Figure 18: Time course of upward graviresponse of hypocotyls of *pcx* plant

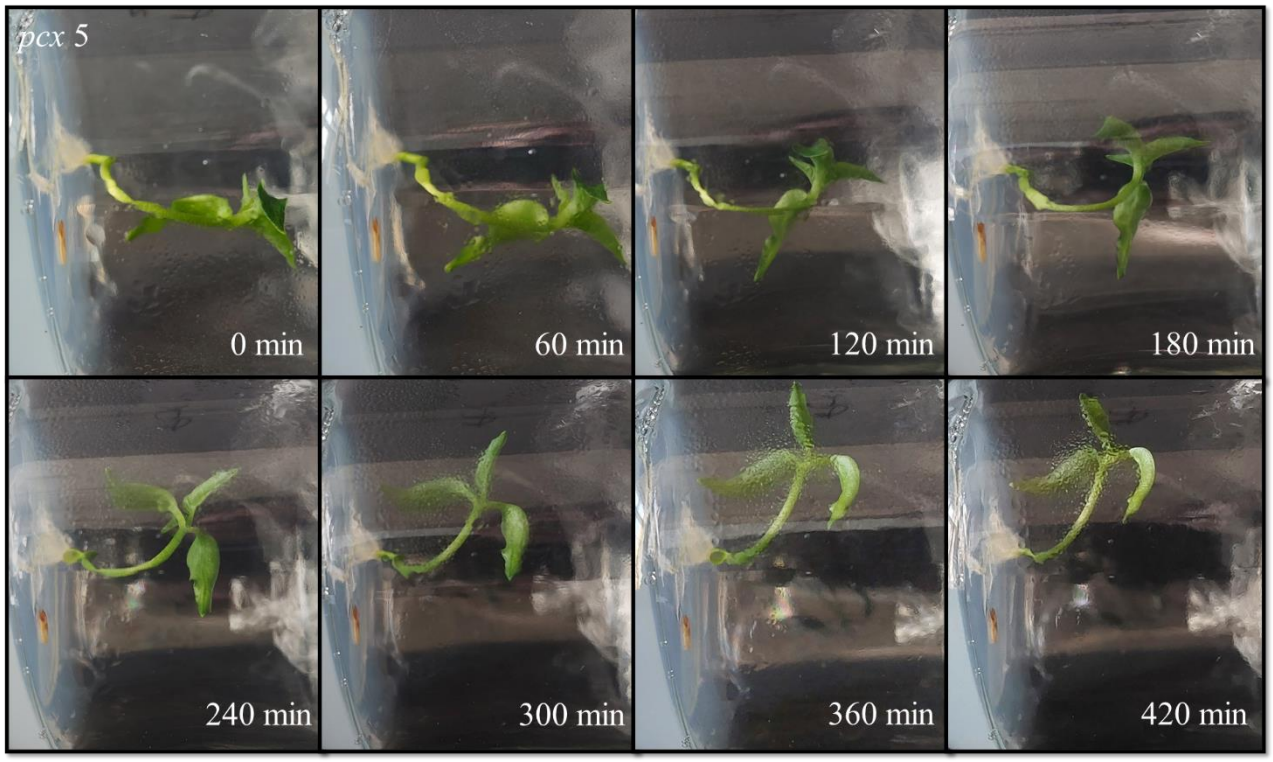


Figure 19: Time course of upward graviresponse of hypocotyls of *pcx* plant

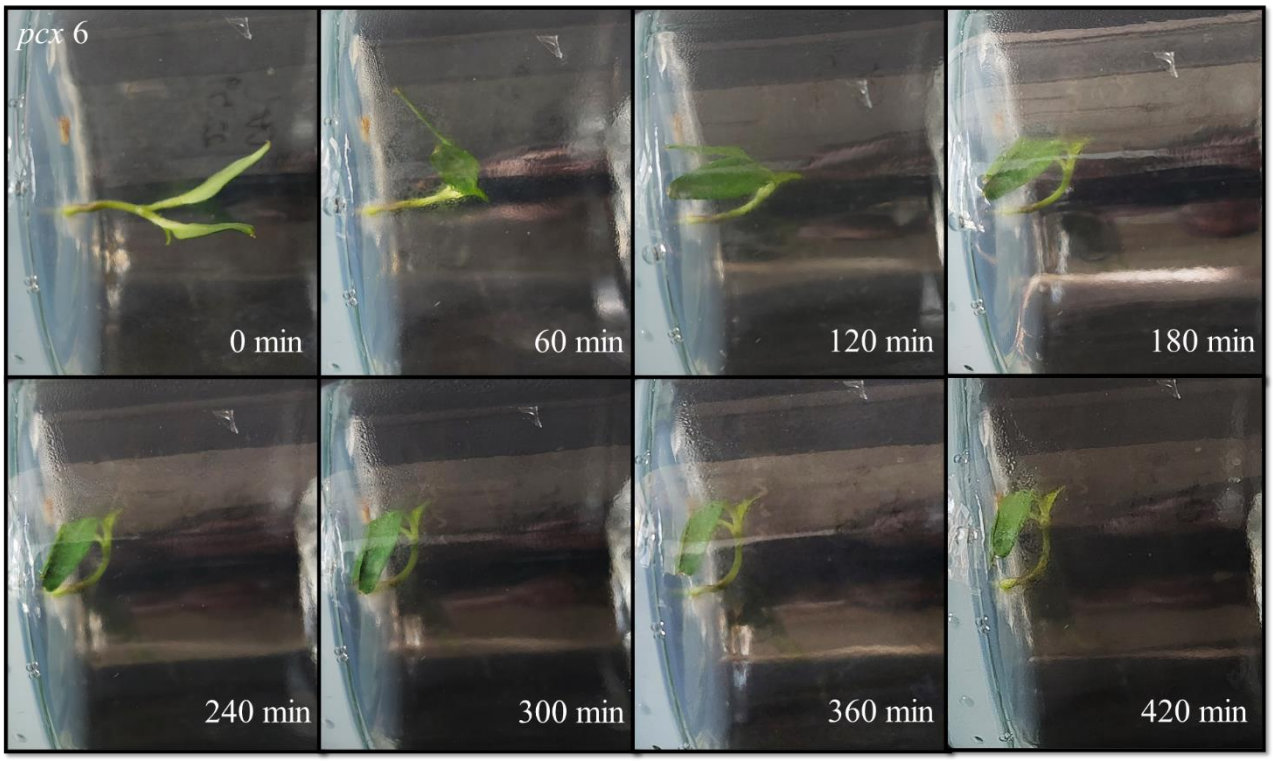


Figure 20: Time course of upward graviresponse of hypocotyls of *pcx* plant

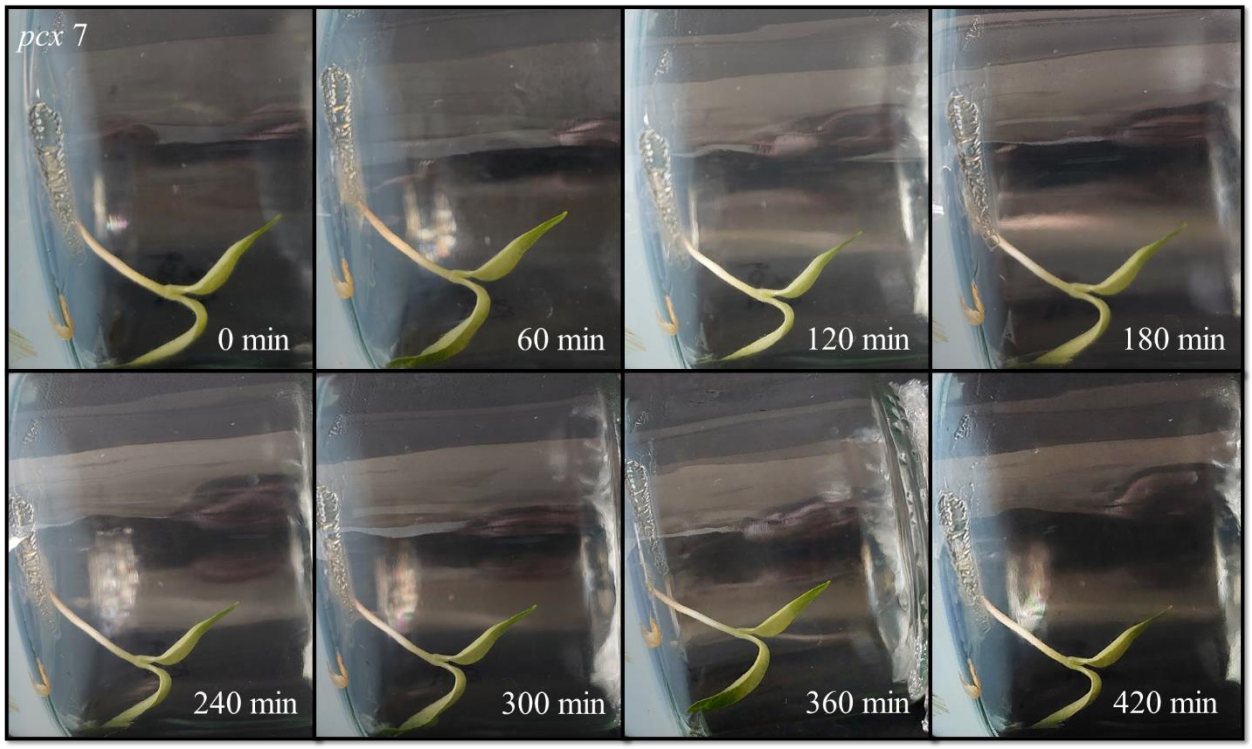


Figure 21: Time course of upward graviresponse of hypocotyls of *pcx* plant

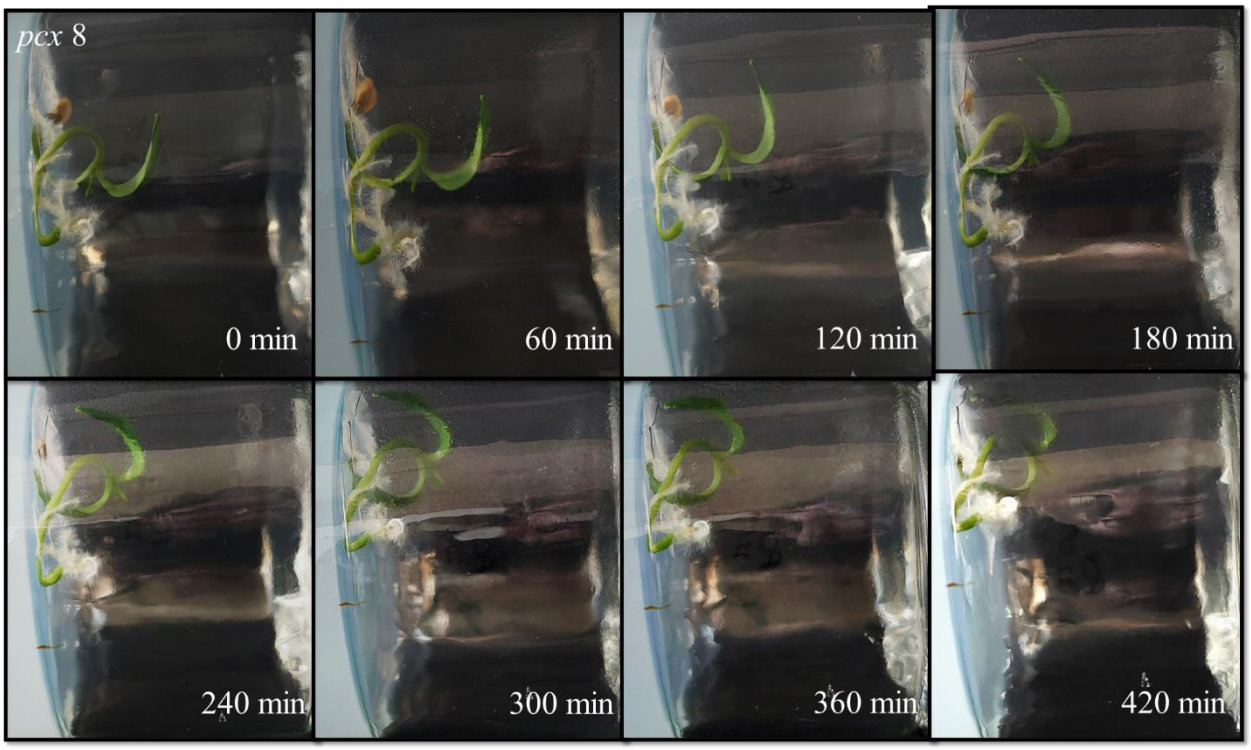


Figure 22: Time course of upward graviresponse of hypocotyls of *pcx* plant

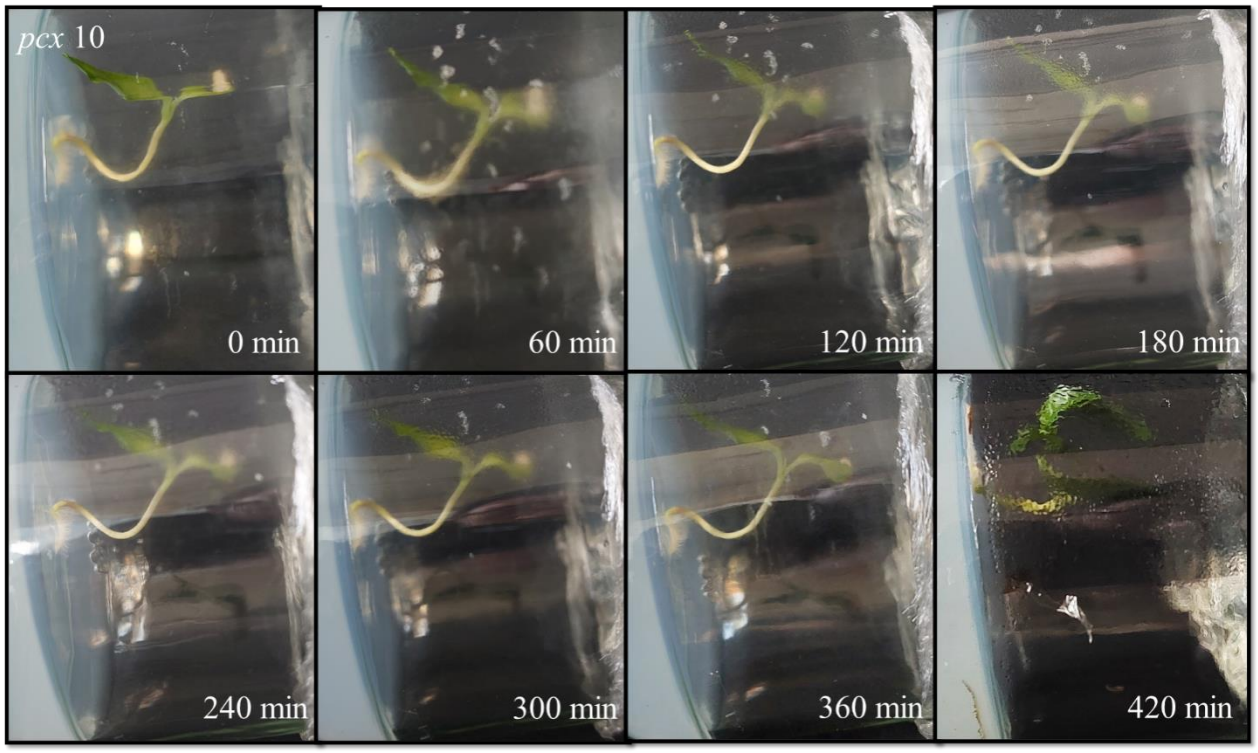


Figure 23: Time course of upward graviresponse of hypocotyls of *pcx* plant

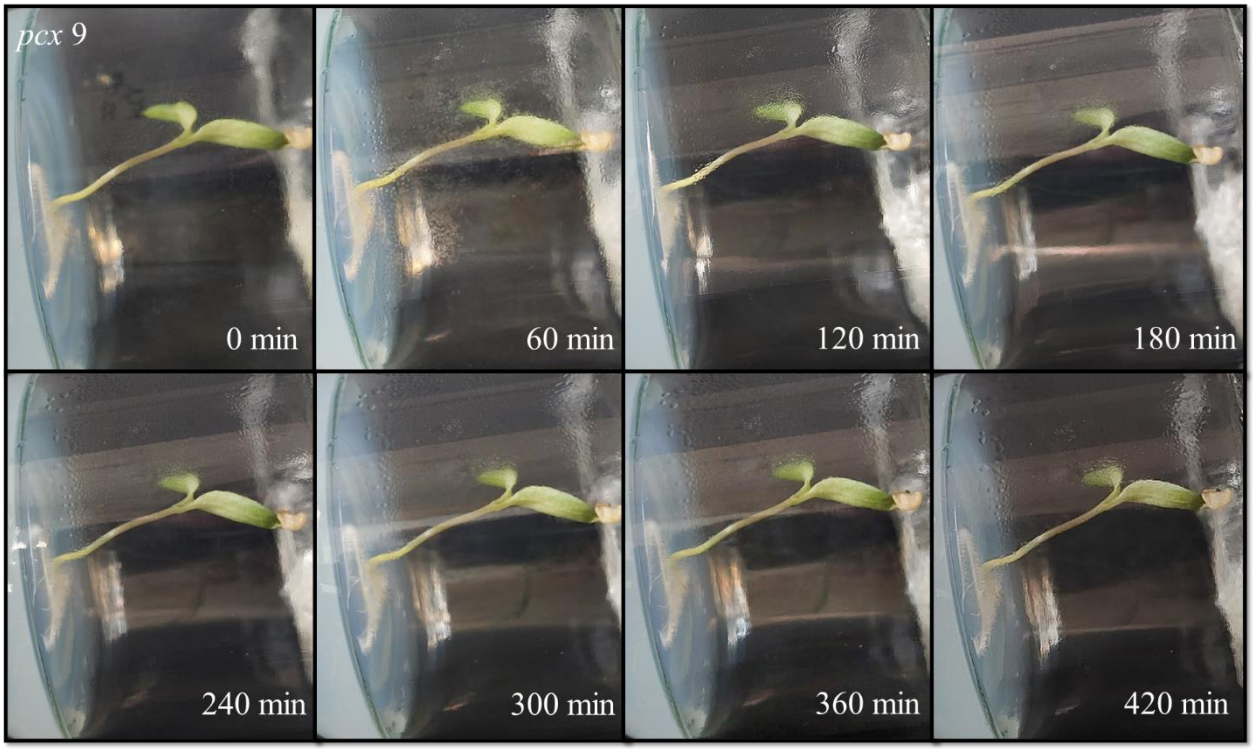


Figure 24: Time course of upward graviresponse of hypocotyls of pcx plant

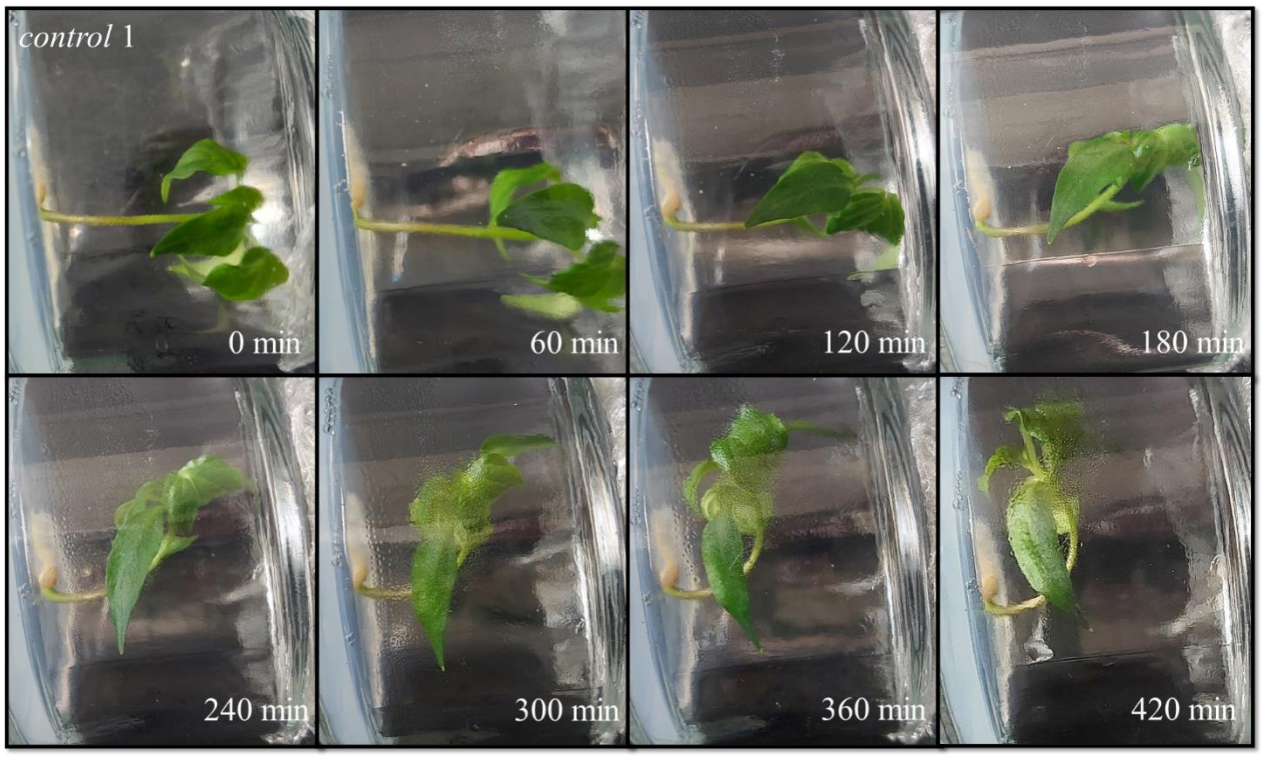


Figure 25: Time course of upward graviresponse of hypocotyls of control plant

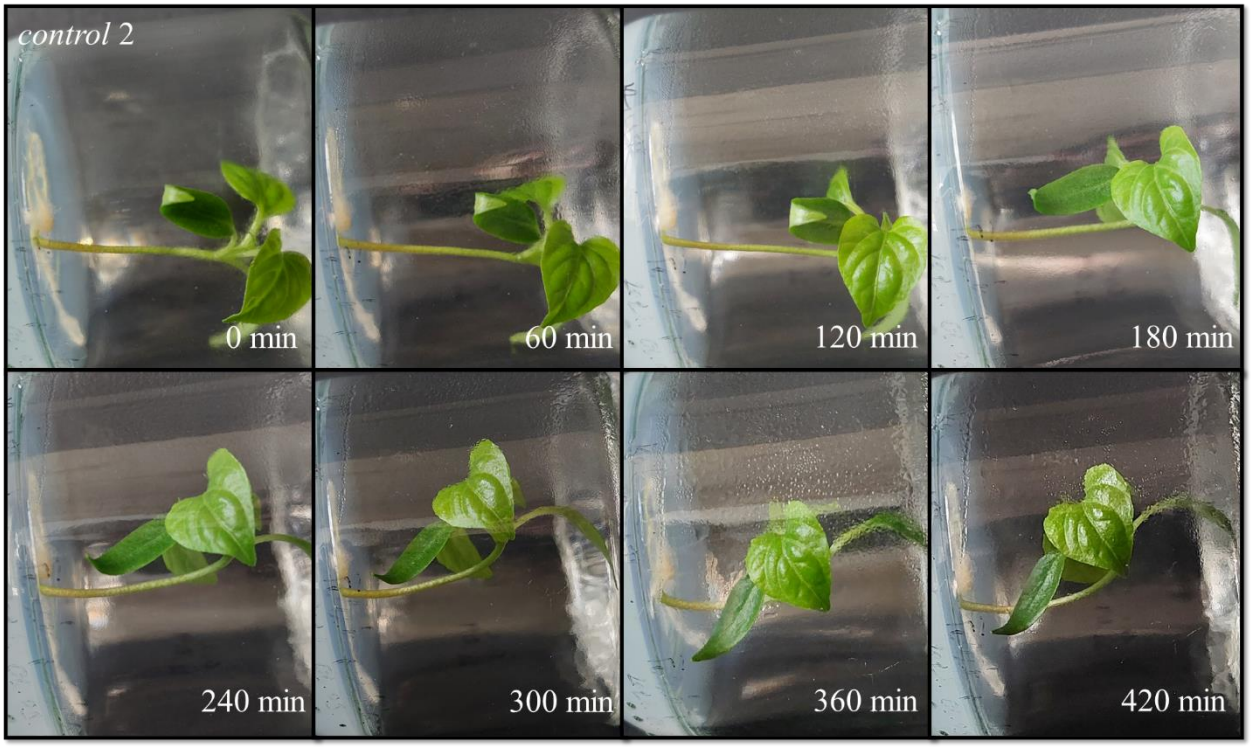


Figure 26: Time course of upward graviresponse of hypocotyls of control plant

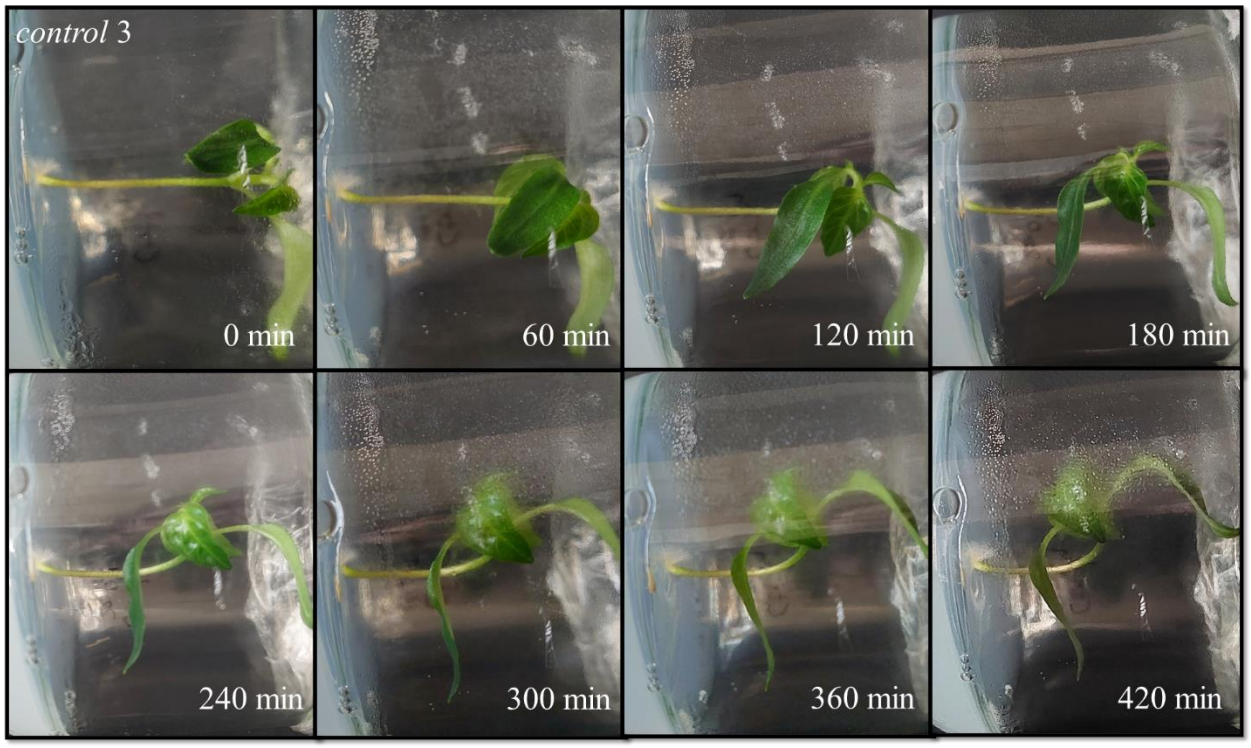


Figure 27: Time course of upward graviresponse of hypocotyls of control plant

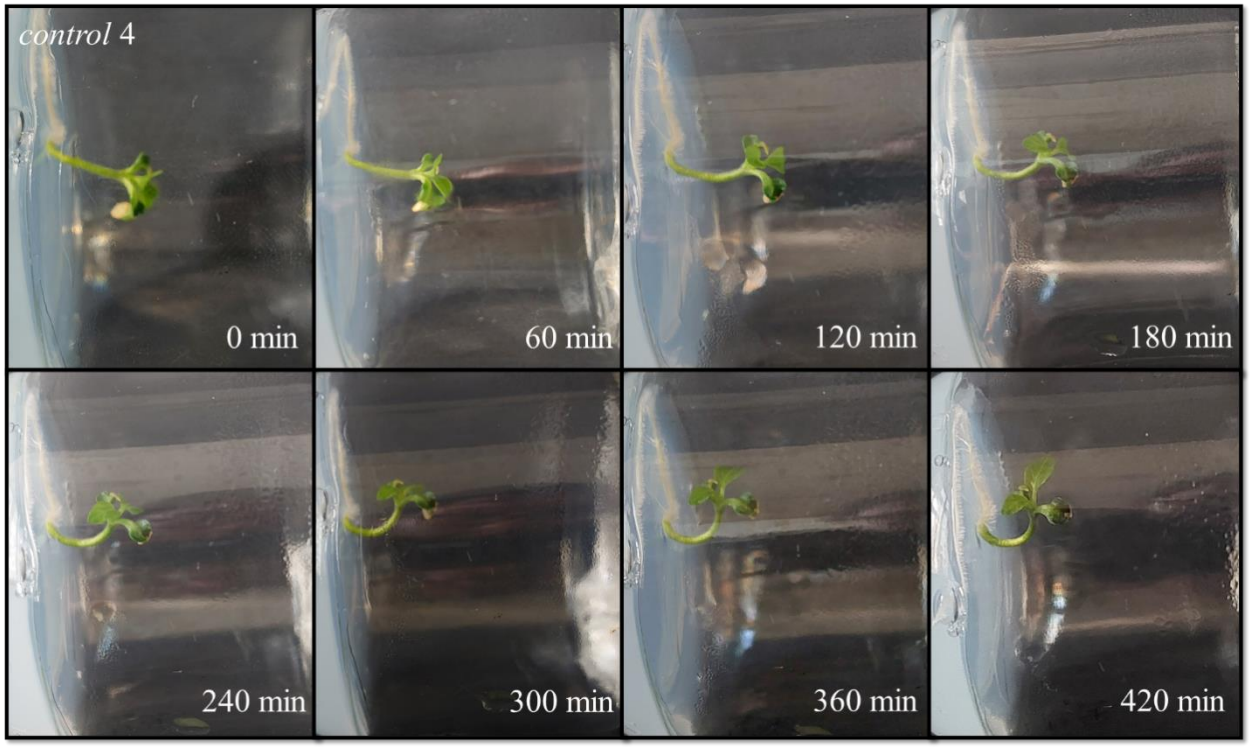


Figure 28: Time course of upward graviresponse of hypocotyls of control

At the end of this experiment, the *pcx* plants were categorized into three groups. The first group contains the *pcx* plants which did not present any abnormal stem growth. The second group contains those plants, which had a slight bending at the beginning, but begin to grow upwards after a while. In the third group we added the plants which were not affected by the gravity at all. The experimental results are presented in figures 15-28. Considering the plants' growth *pcx* 1, 3, 4, 5, 6, 10 belongs to the first group, *pcx* 2 belongs to the second group and *pcx* 7, 8, 9 belongs to the third group. For comparison purposes, the control plants were presented as well. In order to also better visualize this data, a graph was constructed using Microsoft Excel after the images were analysed using the ImageJ analysis software after which the data was generated, and graph plotted.

Table 4: Data generated from images by using an image software

	hypocotyl curvature (°)										
time	pcx1	pcx2	pcx3	pcx4	pcx5	pcx6	pcx7	pcx8	pcx9	pcx10	control
	0	0	0	0	0	0	0	0	0	0	0
0 min	0	-47	-7	8	-23	-4	-48	-80	16	76	0
60 min	6	-38	26	17	41	27	-48	-80	16	76	32
120 min	90	54	44	39	72	39	-48	-74	16	76	56
180 min	90	90	90	45	83	81	-48	-74	16	76	77
240 min	90	90	90	74	83	90	-48	-53	16	90	90
300 min	90	90	90	90	90	90	-48	-53	16	90	90
360 min	90	90	90	90	90	90	-48	-53	16	90	90
420 min	90	90	90	90	90	90	-48	-53	16	90	90

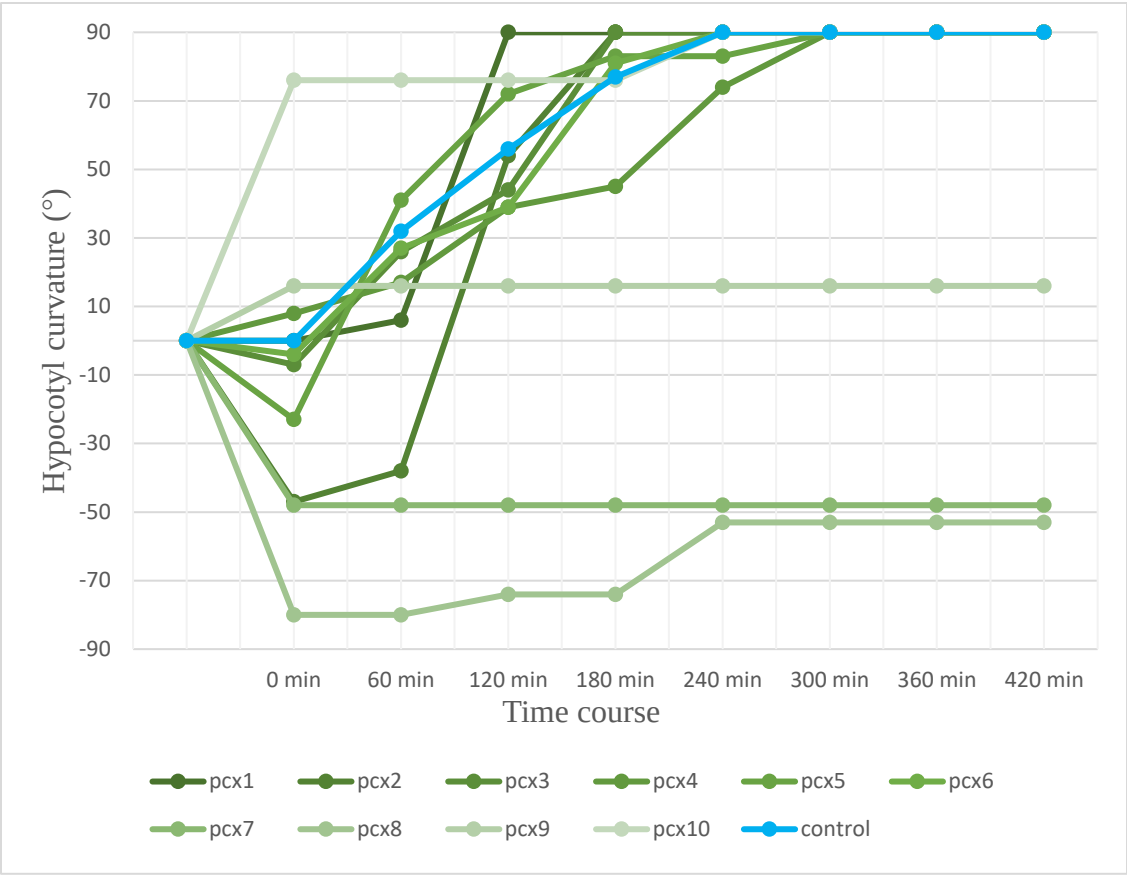


Figure 29: Time course of gravitropic responses of the hypocotyls

Herein on our graph we added the control plant only once since all of them reacted to the gravity after reorientation considerably fast and reached 90° bending.

In summary, it was concluded that *pcx* 1,3,4,5,6,10 plants which had the ability to sense gravity reacted to gravitational pull considerably faster than the other groups were put in group one. From the figures 10,12,13,14,15,19 above, it can clearly be seen that the adjustment began early and reached 90 degrees at 180th minute which was considerably faster than the others. *pcx* 2 (fig. 11) in group 2 begins by bending slightly and then reorient upwards after the 240minute. Group 3 plants ie. pcx 7,8 and 9 (fig. 16,17,18) remained unchanged after several hours of the gravity reorientation. The control plants despite having begun adjusting early, took more time (240 minutes) to reach 90 degrees.

4.3. Lignin content of the hypocotyls

Using Phloroglucinol-Hydrochloric Acid to test the presence of lignin in plant tissues is a common practice. The color change is immediately observable. The experimental result can be seen in figures 30-31-32.

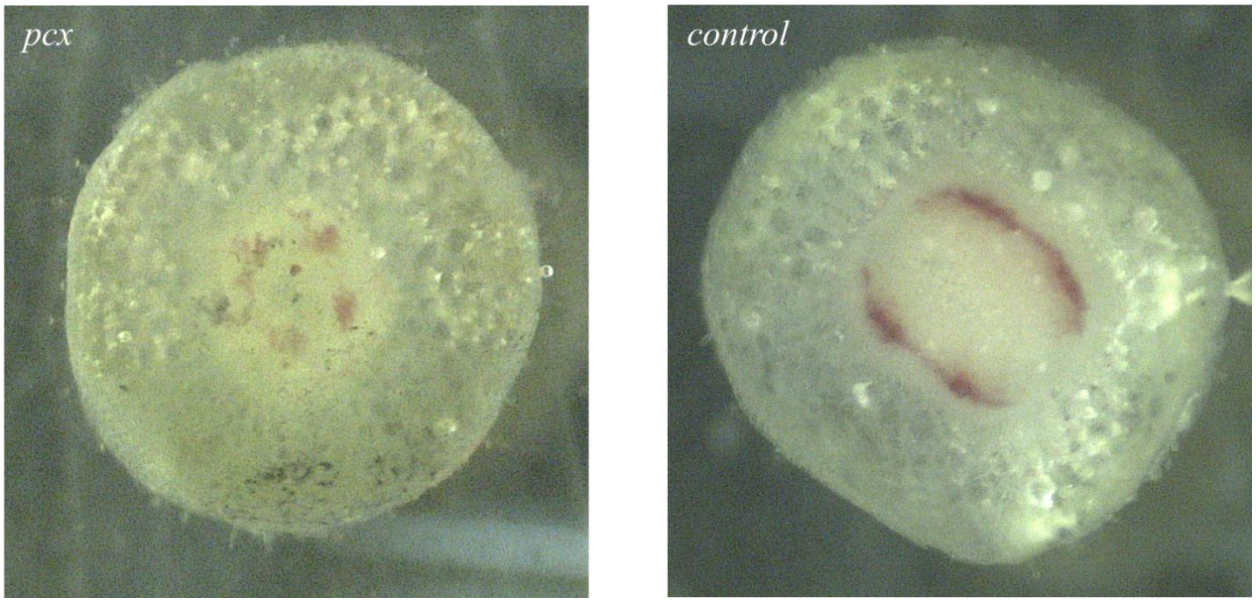


Figure 30-31: *pcx* and control hypocotyl segments stained with Phloroglucinol-Hydrochloric Acid

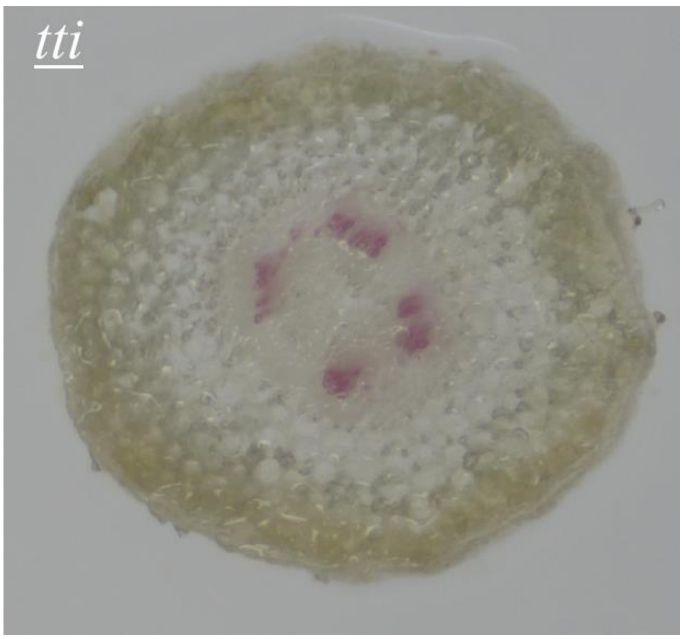


Figure 32: *tti* hypocotyl segment stained with Phloroglucinol-Hydrochloric Acid

As a result of this experiment, some differences between the lignin content staining intensity were observed for the pepper plantlets. *pcx* plants in its early phase had less lignin in its tissues as compared to the *tti* and the control. This result is interesting since considering our observations fully matured *pcx* genotypes grow a very rigid stem. The *tti* plants contained less lignin compared to the control plants.

5. CONCLUSION

Both the root and hypocotyl growth of the procumbent (*pcx*), tortuous internode (*tti*) and the control phenotype were observed in these experiments. The *pcx* plants have a varied spectrum in hypocotyl growth. They were therefore differentiated into three groups. The first group contains the *pcx* plants which did not present any abnormal stem growth. The second group contains those plants, which had a slight bending at the beginning, but started to grow upwards after a while. In the third group were the plants which were not affected by the gravity reorientation. Considering the results above, *pcx* plants 1, 3, 4, 5, 6, 10 belongs to the first group, *pcx* 2 belongs to the second group and *pcx* 7, 8, 9 belongs to the third group. In summary, it can be concluded that *pcx* plants which had the ability to sense gravity reacted to gravitational pull considerably faster than the other groups ie. Group one. It can also clearly be seen that the adjustment began early and reached 90 degrees at 180th minute which was considerably faster than the others. Group 3 plants began by bending slightly and then reorienting upwards after the 240 minute.-decumbent phenotype. Group 2 plants remained unchanged after several hours of the gravity reorientation. The control plants despite having begun adjusting early, took more time (360 minutes) to reach 90 degrees. This was further illustrated in the graph. According to Kiss 2000 and Sack 1991, there are three steps in gravity perception. The first step is the sedimentation of the amyloplasts within the root cap columella cells and the layers of endodermal cells in the hypocotyls and stem. The second step is transduction which involves hormones confirmed by Kaufman et al, 1995 and Muday, 2001. Lastly, is the curvature response that allows the organs to start growth at a specific angle from the gravity vector. The various time differences observed during this experiment could be attributed to a delay in any of these three processes. The group with the unresponsive gravity sensing could be due to the fact that they lack these three processes or the failure of the first step to take place. Several evidence by various authors also suggested that in hypocotyls, gravity can be sensed as they elongate (Boonsirichai et al., 2002).

For checking the phototropic responses, the plants were grown in completely dark environment and in light as well, so it is not presumed their growth habit was because of the lack of gravity sensing. The control plants had its shoot system growing upwards and towards the light and roots growing downwards meaning that the gravity and phototropic vectors were sensed. All plantlets

such as the *tti*, *pcx* and control grew their roots in the direction of gravity. There were no observed differences between the procumbent (*pcx*), tourtous internode (*tti*) and the control phenotype, so we concluded that the mutations only show in the hypocotyl/stem growth. From the results, it can be observed that, the procumbent lines showed normal phototropic responses. Further support came from the observation that, they grew towards the source of light in the phytotron chamber. It can therefore be presumed that the mutant *pcx* plant roots are able to sense gravity, but the hypocotyls face challenges when sensing gravity. Just like gavitropism, phototropism exists in three steps too. The perception of light signal, transduction of signal and the growth response of organs (Grolig et al, 2000). Since our mutants could sense the light signal, it can be concluded that they respond positively to light signals.

Lignin staining techniques involve the use of specific stains that react with the chemical components of lignin, causing it to appear as a distinct color or fluorescence under a microscope or other imaging equipment. For this experiment, phloroglucinol was used. In checking the lignin content, some differences between the *pcx, tti* and the control phenotype. The *pcx* hypocotyl contains less lignin stain as compared to *tti* and the control in this early phase. This shows that lignin is not as localized in *pcx* as compared to the other phenotypes. From growth in the greenhouse, it was observed that fully matured *pcx* genotypes grow a very rigid stem at maturity which was contrary to the result of lignin staining process. The *tti* plants also contained less lignin compared to the control plants.

Lignin staining can provide information on the distribution and localization of lignin in a sample, but it generally does not give a precise measurement of the quantity of lignin. As a result, a quantitative analysis would be required to come to an actual conclusion on the aspect of lignin for these mutants.

6. SUMMARY

Capsicum annuum is a world-wide known and consumed plant. In Hungary bell pepper is important vegetable in traditional cuisine and feeding as well. It is efficient to provide more fruits. Horticulture is a dynamic and a quickly changing sector which needs new ideas, plant materials, and cultivating methods to produce more. The application of mutant traits to breeding lines has become a common method since mutant plants may present some unique properties. that can be useful to create new cultivating methods and breeding lines.

The *pcx* (procumbent) and *tti* (tuortous)pepper is a mutant breeding line from the huge mutant collection of Hungarian pepper breeder Csilléry Gábor. It presents a laying, vine-like growth habit. The laying, procumbent growth-habit might occur because lignin deficiency or any error that might occur the light- or the gravity sensing-reacting process. Understanding the background of this mutant phenotype might help us preparing our own laying plants to use them in different cultivation methods. In this experiment, the mutant plants' reaction to light and gravity were evaluated in *in vitro* culture in comparison to the control line 'Fehérözön'. The results proved that all the plants sensed and reacted to light growing into its direction. In completely dark environment the control plants still reacted to gravitational pull and started to grow upwards while the *pcx* plants show a random hypocotyl growth, the tti plants sensed the gravity but the stem structure was too weak to keep the stems upwards. In all experiments, the roots started to grow in the direction of the gravity. The *pcx* plants were categorized into three groups. *pcx* 1, 3, 4, 5, 6, 10 belonged to the first group, *pcx* 2 belonged to the second group and *pcx* 7, 8, 9 belonged to the third group. The first group contains the *pcx* plants which did not present any abnormal stem growth. The second group contains those plants, which has a slight bending at the beginning, but they start to grow upwards after a while. In the third group we added the plants which were not affected by the gravity at all. This result was further backed by a graph which was obtained by analyzing the images using ImageJ image analyzing software and the graph plotted using Ms Excel.

The lignin content of the hypocotyls was checked using Phloroglucinol-Hydrochloric Acid solution which immediately stains the lignified tissues of the plants. The *pcx* hypocotyl contains less lignin in this early phase as compared to the *tti* and the control. The tti plants also have lower lignin content compared to the control. It was therefore recommended that the experiments be

further studied by making an analytical measurement of the lignin content of the hypocotyls and fully grown stems as well. To also come to a concrete conclusion on the cause of the growth habit of the plants, the genome of the mutants could be sequenced and analyzed. This will help to identify the probable genes responsible for this trait.

7. ACKNOWLEDGEMENT

I am truly grateful to God for granting me the strength to go through my two year study program successfully. I am exceptionally grateful to Him for also seeing me through the writing of my thesis. I profoundly thank the Hungarian Government and the Ministry of Agriculture of Hungary for granting me an opportunity to study in Hungary on the FAO scholarship program. My sincere gratitude to my supervisors Bank Papai and Dr. Aniko Veres for their immense support in helping me write my thesis. This support went a long way to helping me complete this thesis. Many thanks to pepper breeder Csillery Gabor for providing us with the mutant seeds to undertake this experiment. I also would like to thank all faculty members in the Institute of Crop Production who assisted during my study period. Lastly, I would like to thank my parents and siblings for keeping me in their prayers.

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