



In vitro culture and profiling of secondary metabolites in naturally colored cotton (*Gossypium hirsutum* L.)

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Abstract

The cotton (*Gossypium* spp.) plant, which is the leading source of plant-based textile fiber, is also an economically valuable resource for agriculture and many other industries. Some cotton varieties may also have fibers in brown, green and tan tones, aside from the common white. Cotton with this type of fiber development is called ‘Naturally Colored Cotton’ (NCC). Various applications were performed here, including callus induction and propagation, root suspension cultures, and the analysis of secondary metabolite profiles in *in vitro* grown roots and callus tissues of NCC (*Gossypium hirsutum* L. Sarigelin variety). Studies on callus culture have revealed multiple plant growth regulator (PGR) concentrations and combinations with high success rates for callus induction from NCC hypocotyl explants. Among the concentrations tested, MS medium containing 1.0 mg/L IAA and 0.5 mg/L Kinetin was the most effective combination for callus induction. Rooted shoots were successfully propagated from cotyledonary nodes. Following HPLC analyses of white cotton roots, NCC roots, and callus tissues grown *in vitro* revealed both varieties to be rich in phenolic sources such as flavonoids and anthraquinones. Plant tissue culture practices on NCC provide substantial source materials for future biotechnological applications. Therefore, this study is expected to provide valuable data, enabling further research to be conducted on naturally colored cotton.

Key message

Plant tissue culture protocols for naturally colored cottons (NCC) were developed, revealing NCC's significant potential for producing flavonoids and anthraquinones.

Keywords Naturally colored cotton · Callus · *In vitro* propagation · Root suspension · HPLC · Secondary metabolites

Abbreviations

NCC	Naturally colored cotton
PGR	Plant growth regulator
2,4-D	2,4-Dichlorophenoxy acetic acid
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
NAA	Naphthalene acetic acid
BAP	6-Benzylaminopurine

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Introduction

Cotton (*Gossypium* spp.) is a valuable industrial plant used and processed in many industries, especially the textile industry. The cotton plant is cultivated in 78 countries and has a total cultivation area of 32.80 million hectares (Rathinamoorthy and Parthiban 2019). Cotton fiber obtained from the cotton plant, produced primarily in India, China, the United States, Pakistan, and Türkiye, has a significant economic impact and is an exclusive type of fiber for the textile industry (Khan et al. 2020).

Gossypium spp. is commercially important, but its yield is influenced by a range of ecological factors. Temperature is one of the most critical factors for cotton production. Cotton requires temperatures ranging from a minimum of 12–15 °C to a maximum of 20–30 °C. Temperatures below or above these limits adversely affect production efficiency. Drought represents another one of the most significant threats to *Gossypium* spp. cultivation, making it heavily reliant on water

availability. While this plant species exhibits a certain degree of salt tolerance, salinity levels exceeding 7.7 dS m^{-1} also impact growth and development, reducing the yield (Rahman and Farooq 2019).

Among all *Gossypium* species, 90% of the textile industry uses *Gossypium hirsutum*, also known as “upland cotton” or “Mexican cotton” in the agricultural industry, due to its high yield potential and greater adaptability than other *Gossypium* species (Wendel et al. 1992; Khan et al. 2020). In global cotton production, *Gossypium hirsutum* is followed by *Gossypium barbadense*, which accounts for approximately 3–4%. *Gossypium arboreum* and *Gossypium herbaceum* contribute to global cotton production at a combined rate of 2% (Cope 2018). In the textile industry, 50% of fibers are obtained from cotton (Rathinamoorthy and Parthiban 2019).

Dyeing in the textile industry is a process which uses more than 8000 chemicals, presenting a significant concern of pollution for the environment, especially water pollution. According to World Bank data, 17–20% of industrial water pollution is estimated to be caused by dyestuffs used in the textile industry, and 72 of these toxic chemicals have been detected in water sources. Thirty of the toxic chemicals could not be eliminated from the water source. In addition, most of the chemicals used in the textile industry are characterized as toxic and contain factors that are directly or indirectly harmful to human health (Kant 2011). Efforts are being made to develop sustainable and eco-friendly methods for cotton agriculture, though 75% of traditional production methods are still preferred in cotton farming. Therefore, in addition to improvements in production methods, seeking an efficient and eco-friendly raw material that can substitute white fiber cotton has become an urgent requirement (Voora et al. 2023).

Aside from white fibers, *G. hirsutum* can also provide brown, green, and tan fibers by utilizing different pigmentation pathways. Cotton with naturally colored fibers without any synthetic dyeing process is called Naturally Colored Cotton (NCC) (Crews and Hustvedt 2005).

Studies have identified NCCs, primarily as mutants of white cotton derived from *G. hirsutum* and *G. barbadense*. The currently used NCC variants have been developed through selection and recurrent cross-breeding methods (Nagarajan et al. 2022).

The fiber pigmentation in NCC differs from that in white cotton, depending on the flavonoid biosynthesis pathway. In green fibers, lignin and lignan, which are caffeic acid derivatives, are the molecules responsible for pigmentation. In brown fibers, proanthocyanidins (PAs) are the primary pigments responsible for the fiber color. A comparative study conducted on mature white, green, and brown fibers identified the presence of PAs exclusively in brown fibers, while no PAs were detected in green and white fibers (Xiao et al. 2007). Genomic and

transcriptomic analyses on naturally colored cotton have revealed that a series of structural genes involved in the flavonoid biosynthesis pathway which are upregulated in brown fibers (Noumkina et al. 2024).

As in other industries, the textile industry is also searching for production methods that are less harmful to the environment. Along with NCC, various eco-friendly production methods called green textiles have emerged in the textile industry (Zhu et al. 2006). NCC varieties present an eco-friendly alternative to white cotton by eliminating the chemical dyeing process in the textile industry, while also being less harmful to human health. Besides, NCCs are more resistant to biotic stress factors such as agricultural pests and abiotic stress factors such as salinity and drought than white cotton. Therefore, NCCs are a more suitable alternative for agriculture in terms of production (Rathinamoorthy and Parthiban 2019).

A study comparing NCC varieties and white cotton, regarding fiber processing cost, determined that the fabric obtained from NCC varieties was 2.9 times less costly than white cotton (Gülümser 2016). Also, fabrics produced from NCC varieties had a high ultraviolet protection factor (UPF), and the UPF value remained high even after repeated washing (Parmar et al. 2006).

Although NCC varieties have the advantages described above, crop yields are lower than white cotton's, making them economically disadvantageous. Fibers of NCC varieties are generally shorter, less resistant, weaker, and thicker than white cotton, causing difficulties in processing industrially. Therefore, molecular biotechnological research on fiber quality is needed for NCC to reach the level of white cotton commercially (Abbasi 2019). However, it may be challenging for traditional breeding methods to provide significant improvements on issues such as color spectrum and low fiber yield, which are critical for NCC (Wen et al. 2024).

At this point, one of the most critical steps for new studies on NCCs is the rapid and sufficient production of tissue sources to be used in experimental setups. Therefore, conducting more studies on plant tissue culture methods that have not yet been optimized for NCCs are necessary.

Among the types of plant tissue culture methods, callus cultures have been used as experimental material in many studies, such as obtaining a whole plant from any plant organ efficiently, genetic analyses to understand the responses to stress conditions, and production of economically valuable secondary metabolites on large scales (Efferth 2019). Although various physical conditions (temperature, humidity, light, etc.) are essential factors for callus formation, the condition with the most significant effect is the content of the callus induction medium. During callus induction, auxin and cytokinin derivatives can be used alone or in combination as plant growth regulators (Zhu et al. 2006).

Micropropagation is an important tool for the large-scale production of genetically stable and pest-free clonal plantlets. Reducing the cost of the production process and increasing efficiency and production speed are the main advantages of micropropagation (Cardosa et al. 2018). Another type of *in vitro* culture method is root suspension cultures, where roots are suspended using liquid media, shaken at a certain rpm, and usually supplemented with an appropriate auxin derivative. Root suspension cultures offer practical advantages and exhibit more genetic stability than other tissue culture methods. Therefore, they are suitable for germplasm preservation of certain species. Root suspension cultures are frequently used in studies investigating the production of various proteins and secondary metabolites (Bhaita 2015).

Plant secondary metabolite production, an essential extension of plant tissue culture studies, constitutes an important branch of plant biotechnology. Flavonoids are secondary metabolites consisting of a benzopyrone ring with phenolic or polyphenolic groups at different positions. More than 10,000 flavonoid compounds have been isolated and identified so far. Most flavonoids are recognized as therapeutic agents (Ullah et al. 2020). Quinones, another phenolic compound group, are a class of organic compounds characterized by a cyclic diketone structure and anthraquinones are an important subgroup of this class (Malik and Müller 2016). Anthraquinone derivatives have been identified as the largest group of natural quinones. They constitute the largest group of natural pigments with approximately 700 compounds, mainly emodin, physcion, catenarin, and rhein. Anthraquinones have been used as pigments in many industries, including cosmetics, food, and textile industries, for many years, due to their coloring properties and have received significant attention. Anthraquinone derivatives exhibit a wide range of pharmacological activities in modern medicine, including anticancer (Malik et al. 2021), anti-inflammatory (Wang et al. 2020), antiarthritic (Kshirsagar et al. 2014), antifungal/antibacterial (Raghuveer et al. 2023), antiviral (Parvez et al. 2019) and neuroprotective effects, and there is an increasing interest in identifying new anthraquinone sources (Malik and Müller 2016).

This study developed procedures for initiating callus cultures, root suspension cultures, and *in vitro* propagation using the NCC Sarigelin variety, which has an important potential for the environment and a sustainable textile industry. HPLC analyses were also carried out using callus and root suspension cultures to investigate the secondary metabolite profiles of NCC. These procedures aim to overcome the lack of information in the literature concerning NCC tissue culture methods. The data obtained in this study will be valuable for biotechnological applications aimed at improving the industrially disadvantageous traits of NCC. Additionally, this study aims to reveal the potential of NCC varieties regarding flavonoid and anthraquinone metabolite

contents with high economic value by obtaining new data on secondary metabolite profiles.

Material and methods

Plant materials and seed germination

Seeds of Harem II variety with white fiber color and Sarigelin variety with light brown fiber color were obtained from the Cotton Research Institute Directorate of the Republic of Türkiye, Ministry of Agriculture and Forestry.

After germination, hypocotyls of 7 day-old NCC Sarigelin variety were used for callus induction; cotyledonary nodes were used for *in vitro* shoot formation and rooting, and roots of 7-day-old plantlets were used to establish root suspension cultures. The callus and root tissues obtained after these processes were used for secondary metabolite analyses. The experimental procedures are detailed in the following sections. The acquisition of explants and the analyses performed are shown in Fig. 1.

The sterilization method developed by Ahmed et al. (2020) for white fiber cotton was modified for surface sterilization of NCC cotton seeds. Sarigelin seeds were washed in ethanol (70%) for 1 min, and ethanol was removed. Seeds were incubated in H₂O₂ (10%) at 120 rpm for 2 h. H₂O₂-treated seeds were rinsed with sterile dH₂O 3 times for 5 min. Seeds were peeled down to the embryo with sterile forceps and scalpel and inoculated on MS medium containing 0.9% agar (Murashige and Skoog 1962). Seeds were germinated for 7 days in a plant growth cabinet (Sanyo, MLR-352H) at 25 ± 2 °C, 75 µmol.m⁻² s⁻¹ light intensity and 16/8 h (light/dark) light cycle.

Callus induction

The medium content used for callus induction from NCC hypocotyl explants was determined by modifying the plant growth regulators (PGRs) and their concentrations in MS basal medium according to previously used media for white fiber cotton (Davidonis and Hamilton 1983; Pederson 1986; Wu et al. 1998; Zhu et al. 2006; Michel et al. 2008 and Kumar et al. 2015). The pH of the MS medium (MS Basal Salt Mix and 3% sucrose) was adjusted to 5.6–5.8. The medium was sterilized by autoclaving (121 °C, 1.2 atm, 15 min) after adding Agar (0.9%). MS vitamin solution (1.0 mg/L) and 0.22 µm filter-sterilized PGR combinations with respective concentrations in Table 1 were added to the sterile media.

Indole-3-acetic acid (IAA) (Sigma, 60K3860), indole-3-butyric acid (IBA) (Sigma, I5386-1G), naphthalene acetic acid (NAA) (Sigma, 53H4600) and 2,4-dichlorophenoxy acetic acid (2,4-D) (Sigma, 080M0115V),

Fig. 1 Explant sources and methods used throughout this study. In the figure, tissues are marked with dashed frames, methods and processes with solid black frames, and sequences of processes with black arrows. Seven-day-old plantlets were utilized: source tissues were obtained, hypocotyls were used for callus induction, cotyledonary nodes were used for shoot and root regeneration, respectively, and root tissues were used to establish root suspension cultures

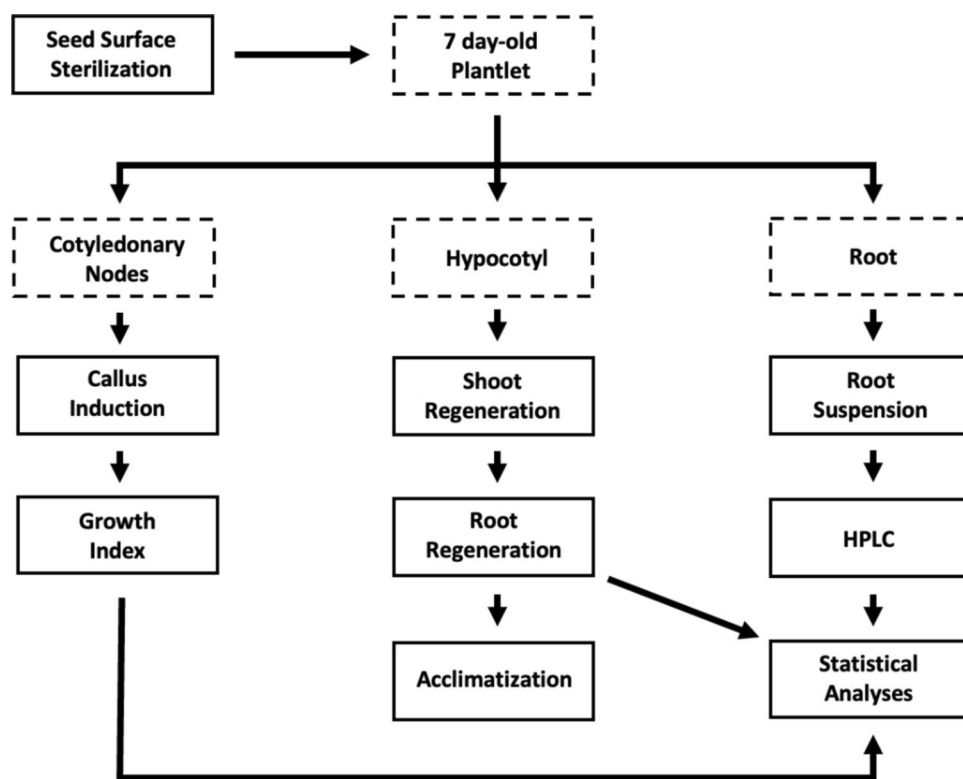


Table 1 Plant growth regulator combinations used for callus induction from NCC

PGR combinations	Plant growth regulators (mg/L)					
	2,4-D	NAA	IAA	IBA	BAP	Kinetin
PGR 1.1	1.0	–	–	–	–	0.5
PGR 1.2	0.5	–	–	–	–	1.0
PGR 2.1	0.5	–	–	–	1.0	–
PGR 2.2	1.0	–	–	–	0.5	–
PGR 3.1	–	–	0.5	–	–	1.0
PGR 3.2	–	–	1.0	–	–	0.5
PGR 4.1	–	–	0.5	–	1.0	–
PGR 4.2	–	–	1.0	–	0.5	–
PGR 5.1	–	–	–	0.5	1.0	–
PGR 5.2	–	–	–	1.0	0.5	–
PGR 6.1	–	–	–	0.5	–	1.0
PGR 6.2	–	–	–	1.0	–	0.5
PGR 7.1	–	0.5	–	–	–	1.0
PGR 7.2	–	1.0	–	–	–	0.5
PGR 8.1	–	0.5	–	–	1.0	–
PGR 8.2	–	1.0	–	–	0.5	–

6-benzylaminopurine (BAP) (Sigma, 031M0234V), and kinetin (Sigma, 22K2378) were used as PGRs in callus induction media.

Hypocotyls of 7-day-old germinated Sarigelin variety plantlets were cut into approximately 1 cm in diameter. These pieces were cut in half with a scalpel vertically to create a wound in the tissue and inoculated in the MS medium

supplemented with PGRs. Callus cultures were incubated in plant growth cabinet with previously described conditions and subcultured every 21 days into fresh medium with the same content as the initial media. The callus induction ratio was calculated using the following equation:

Callus induction ratio (%) = (Total explants inducing callus/Total explants cultured) × 100% (Islam et al. 2004).

Medium specific callus growth index

Considering callus induction rates, some selected PGR combinations were used to calculate medium-specific callus growth indexes. 90 day-old calli of the Sarigelin variety was inoculated in growth index media at 0.2 g in 3 technical replicates. Callus cultures were incubated for 21 days in the same conditions as in the callus induction step. After 21 days, the medium-specific growth index was calculated using the following equation:

Growth Index (Fold Growth) = (Final weight of callus tissue (g) — Initial weight of callus tissue (g)) / Initial weight of callus tissue (g) (Sahraroo et al. 2014).

In vitro propagation of NCC

In this study, the methods previously used by Özyiğit and Gözükırmızı (2008) and Divya et al. (2008) were modified and applied to establish *in vitro* propagation of NCC.

The roots and leaves of the 7-day-old Sarigelin variety were cut from cotyledonary nodes. The cotyledonary nodes were cultured in MS medium for 21 days in a plant growth cabinet for shoot and leaf development.

The 30-day-old explants showing shoot and leaf development were transferred to MS medium containing PGR 6.2, ½ MS medium, and MS medium supplemented with 1.0 mg/L IBA for rooting. MS Medium was used for rooting control. Explants cultured for 30 days were statistically analyzed for lateral root development, root length, and stem length.

Plantlets showing root development were transferred into sterile soil containing 10% perlite with plastic protectors for acclimatization. The plants were irrigated with a 1:1 (v:v) mixture of liquid MS medium and water.

Root suspension cultures

The roots of 7-day-old Sarigelin and Harem II varieties were separated from the stem, and 0.3 g of root tissue was transferred to liquid MS (control) and liquid MS medium containing PGR 6.2 for investigate the effect of PGRs on root tissue growth rate and secondary metabolite content. The white cotton (Harem II) variant was included in the study to investigate the effect of genotypic diversity on root tissue growth rate and secondary metabolite content in comparison to the naturally colored cotton (Sarigelin) variant. Root tissues were incubated at 24 °C, 120 rpm for 21 days in a dark shaking incubator. At the end of the culturing period, root tissues were photographed for morphological analyses, frozen by liquid nitrogen, and stored at – 80 °C for further analysis. In this study, root suspension cultures were established by modifying the method used by Kausch and Horner (1984). Root growth indexes were calculated using the same equation from the callus growth index calculations.

Methanol extraction and HPLC analyses

Methanol extraction and HPLC analyses were conducted on both Harem II and Sarigelin cotton varieties to compare their secondary metabolite profiles. All procedures were performed on roots from suspension cultures as well as on callus tissues. These analyses investigated the effects of cotton variety, tissue source, and medium composition on the secondary metabolite profiles.

Root and callus tissues of 0.5 g were powdered in a mortar and pestle using liquid nitrogen. Methanol (20 mL/g tissue) was added to the powdered calli and roots. The tissue/methanol mixture was subjected to probe-assisted ultrasonication (Bandelin, SONOPULS) for 15 min, 5 × 10% cycle, and 60% power conditions on dry ice to maintain the temperature. After ultrasonication, the samples were centrifuged at 4100 rpm for 15 min at room temperature. After centrifugation, 1 mL of the supernatant was transferred to fresh tubes. Methanol was removed from the extracts by evaporation using a centrifugal evaporator.

HPLC analyses were performed to determine the secondary metabolite profiles of Harem II and Sarigelin from *in vitro*-grown roots, and callus tissues of Sarigelin incubated in PGR 3.2, PGR 4.2, and PGR 5.2. These analyses were performed using Shimadzu® high-performance liquid chromatography system with pump (LC-10AD VP), diode array detector (SPD-M20A), column oven (CTO-10AS), autosampler (SIL-20A), and degassing unit (DGU-20A).

Table 2 indicates the chromatographic conditions and flow system program (Akpınar et al. 2024; Baştumur et al. 2024) under which the analyses were performed.

Table 2 Conditions of the chromatographic processes

Column	ZORBAX Eclipse plus C18 (25 cm × 4.6 mm, 5 µm)	
Flow rate	1.0 mL/min	
Wavelength	210 nm	
Injection volume	20 µL	
Column temperature	25 °C	
Injection time	45 min	
Flow system	Gradient flow	
Mobile phase A	dH ₂ O + 0.5% phosphoric acid	
Mobile phase B	Methanol	
Flow system program	Time (min)	Volume (B)
	0–2	20%
	2–17	50%
	17–35	90%
	35–45	20%
	45	Stop

Statistical analyses

GraphPad Prism® 9.2.0 (Demo) statistical software was used to visualize the data obtained from statistical analyses throughout the experiment. GraphPad Prism® (v9.2.0), Microsoft Excel (v16.58), and IBM SPSS Statistics (v29.0.1.0) (Demo) were used to process the raw data obtained in the experimental steps and to perform statistical significance tests. Each statistical test was performed as at least three biological replicates.

Results and discussion

Naturally colored cottons are an alternative resource for the textile industry due to its environmentally friendly production process. However, optimizing NCC tissue culture conditions is critical for carrying out biotechnology-based studies to increase yield. From this standpoint, noteworthy data have been provided in this study for further research to be carried out.

Harem II variety with white fiber and the Sarigelin variety with light brown fiber were used throughout the study. These two varieties allowed comparative results, especially regarding secondary metabolite profiles. The seeds of Sarigelin and Harem II varieties sterilized by H_2O_2 had 100% germination efficiency and 0% contamination rate in the MS medium. Therefore, this H_2O_2 -based sterilization method was determined as suitable for the seed sterilization of Sarigelin and Harem II varieties.

Effect of different PGR combinations on callus induction and morphology

To examine the effect of different PGRs on callus induction in the NCC Sarigelin variety, 16 different media were used, each containing various concentrations and types of PGRs. As a result of statistical analyses performed on the 21st day of callus induction, effects of the media contents given in Table 1 were significantly different in terms of callus induction ratios.

Considering these data, PGR 3.2, PGR 4.2, and PGR 5.2 were determined as the three most effective callus induction media for the NCC Sarigelin variety. PGR 3.2, PGR 4.2, and PGR 5.2 showed callus induction rates of $93.33\% \pm 6.67$, $83.33\% \pm 3.33$, and $85.83\% \pm 1.44$, respectively. Combinations using IAA and IBA as auxins and BAP and Kinetin as cytokinins resulted in higher callus induction ratios. The comparison of callus induction media with each other regarding induction ratios is shown in Table 3.

Sudheer et al. (2022) reported various physical conditions (temperature, humidity, light, etc.) as important factors for plant tissue culture. However, it was also stated that the

Table 3 Callus induction percentages of different PGR combinations

PGR combinations (mg/L)	Callus induction ratios
1.0 2,4-D + 0.5 Kin (PGR 1.1)	$66.08\% \pm 4.06^{d,e,f}$
0.5 2,4-D + 1.0 Kin (PGR 1.2)	$31.11\% \pm 3.85^g$
0.5 2,4-D + 1.0 BAP (PGR 2.1)	$56.63\% \pm 3.35^f$
1.0 2,4-D + 0.5 BAP (PGR 2.2)	$70.71\% \pm 1.00^{c,d,e}$
0.5 IAA + 1.0 Kin (PGR 3.1)	$77.22\% \pm 2.55^{b,c}$
1.0 IAA + 0.5 Kin (PGR 3.2)	$93.33\% \pm 6.67^a$
0.5 IAA + 1.0 BAP (PGR 4.1)	$64.51\% \pm 1.03^{e,f}$
1.0 IAA + 0.5 BAP (PGR 4.2)	$83.33\% \pm 3.33^b$
0.5 IBA + 1.0 BAP (PGR 5.1)	$61.67\% \pm 2.35^{e,f}$
1.0 IBA + 0.5 BAP (PGR 5.2)	$85.83\% \pm 1.44^{a,b}$
0.5 IBA + 1.0 Kin (PGR 6.1)	$70.00\% \pm 8.82^{c,d,e}$
1.0 IBA + 0.5 Kin (PGR 6.2)	$67.77\% \pm 5.09^{d,e}$
0.5 NAA + 1.0 Kin (PGR 7.1)	$69.36\% \pm 2.44^{c,d,e}$
1.0 NAA + 0.5 Kin (PGR 7.2)	$65.55\% \pm 5.09^{d,e,f}$
0.5 NAA + 1.0 BAP (PGR 8.1)	$62.22\% \pm 9.76^{e,f}$
1.0 NAA + 0.5 BAP (PGR 8.2)	$74.44\% \pm 5.09^{c,d}$

*The difference between the values with the same letter in the callus formation percentage column is insignificant according to the DMR test ($p < 0.005$)

primary parameter affecting the response in tissue culture is PGR. In parallel with the results of this study, the PGR content of the medium we used for callus induction in NCC was indeed an important parameter in terms of callus induction rates.

In previous studies on white fiber cotton, MS medium supplemented with 0.1 mg/L 2,4-D + 0.5 mg/L Kinetin (Michel et al. 2008) and 2.0 mg/L 2,4-D + 2.4 mg/L Kinetin (Kumar et al. 2015) were found to have the highest callus induction ratios. Accordingly, combinations using 2,4-D as auxin and Kinetin as cytokinin were reported as optimum for callus induction. However, the optimum PGR combinations determined in this study differed from the previous literature. The combination of IAA and Kinetin (PGR 3.2) with the highest callus induction rate in our study was previously characterized as the optimum PGR combination for callus induction in different plants such as *Capsicum annuum* L. (El Kabby et al. 2015) and *Vernonia anthelmintica* (Rajan et al. 2020) furthermore at different concentrations. We also determined that in the media with the highest induction percentages (PGR 3.2, PGR 4.2 and PGR 5.2), cytokinin concentrations were lower than auxins.

In this study, media containing 2,4-D had callus formation percentages of 66.08% (PGR 1.1), 31.11% (PGR 1.2), 56.63% (PGR 2.1), and 70.71% (PGR 2.2). In addition, the combination with the lowest percentage of callus formation was determined as the PGR 1.2 combination which also contains 2,4-D. These media had significantly lower callus induction rates than the PGR 3.2 combination with the

highest callus induction rate. Previous studies by Mishra et al. (2003) on white fiber cotton and Zhu et al. (2006) on green fiber NCC identified 2,4-D as essential for callus formation. However, in our study, 2,4-D was not critical to callus induction for light brown fibered Sarigelin variety.

Callus development was photographed and examined at 21 day intervals for morphological analysis. After the second subculture interval, the morphology of the induced callus was examined, and PGR combinations resulted in differences of callus texture and color. In terms of texture, friable and compact calli were obtained in different tested media. In addition, calli colors in different heterogeneity, including brown, green, and white, were observed throughout the study. This was similar to previous studies on white fiber cotton (Michel et al. 2008; Kumar et al. 2015) and green fiber NCC (Zhu et al. 2006).

Morphological differences of callus tissues induced using different combinations of PGRs are shown in Fig. 2.

Indirect adventitious organ development was also observed on calli in PGR 3.1, PGR 3.2, PGR 6.1, and PGR 6.2. In particular, 53.11% of the callus induced in PGR 6.2 developed an indirect adventitious root. Therefore, it was determined that a high IBA/low cytokinin PGR combination was suitable for *in vitro* adventitious root development from callus. This result is similar to previous work where only auxin derivatives in a root induction step on white fiber cotton were used, and the combination with the highest root tissue formation was MS medium supplemented with 1.0 mg/L IBA (Divya et al. 2008).

In 16 different PGR combinations used, different yield and morphology results were obtained. The reason for this situation was determined to be the genotype-dependent

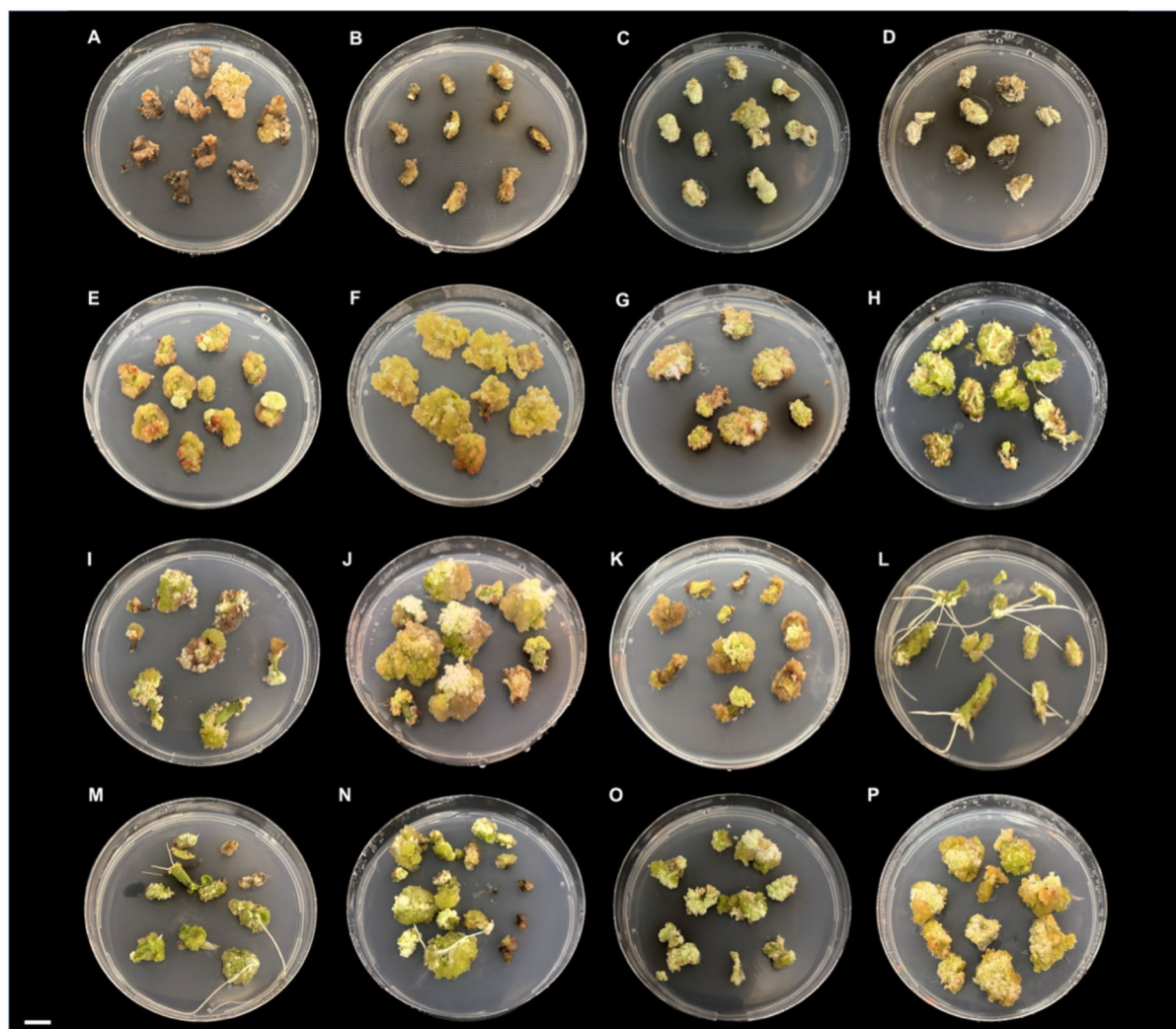


Fig. 2 Callus morphology of NCC Sarigelin variety induced by different PGR combinations on the 42nd day of culturing. **A:** PGR 1.1, **B:** PGR 1.2, **C:** PGR 2.1, **D:** PGR 2.2, **E:** PGR 3.1, **F:** PGR 3.2, **G:**

PGR 4.1, **H:** PGR 4.2, **I:** PGR 5.1, **J:** PGR 5.2, **K:** PGR 6.1, **L:** PGR 6.2, **M:** PGR 7.1, **N:** PGR 7.2, **O:** PGR 8.1, **P:** PGR 8.2

variability of plant responses in plant tissue culture, as previously stated in the literature (Michel et al. 2008). Detailed information on the morphological variations is given in Table 4.

Effect of different PGR combinations on callus proliferation

PGR 3.2, PGR 4.2, and PGR 5.2, with the highest callus induction values from the callus induction step, were tested to understand the effects on callus proliferation. As a result of the analysis at the end of the 21st day, PGR 3.2, PGR 4.2, and PGR 5.2 significantly differed regarding growth indexes. PGR 4.2 and PGR 5.2 showed 1.387 ± 0.110 and 1.177 ± 0.112 -fold growth, respectively, resulting in significantly more proliferation than PGR 3.2, which showed 0.703 ± 0.093 -fold growth ($p < 0.005$). In contrast, no significant difference was observed between MS media supplemented with PGR 4.2 and PGR 5.2. Growth index values of PGR 3.2, PGR 4.2, and PGR 5.2 are shown in Fig. 3.

The PGR 4.2 used in this study resulted in higher proliferation and was found to be an ideal proliferation medium for the callus tissues of Sarigelin. In addition, BAP has been used as a cytokinin derivative, significantly increasing callus proliferation compared to kinetin. In a previous study on white fiber cotton by Kumar et al. (2015), the combination of MS medium supplemented with 0.1 mg/L 2,4-D + 0.6 mg/L 2iP showed the highest callus proliferation value with 0.89 ± 0.20 -fold-growth. The PGR 4.2 combination used

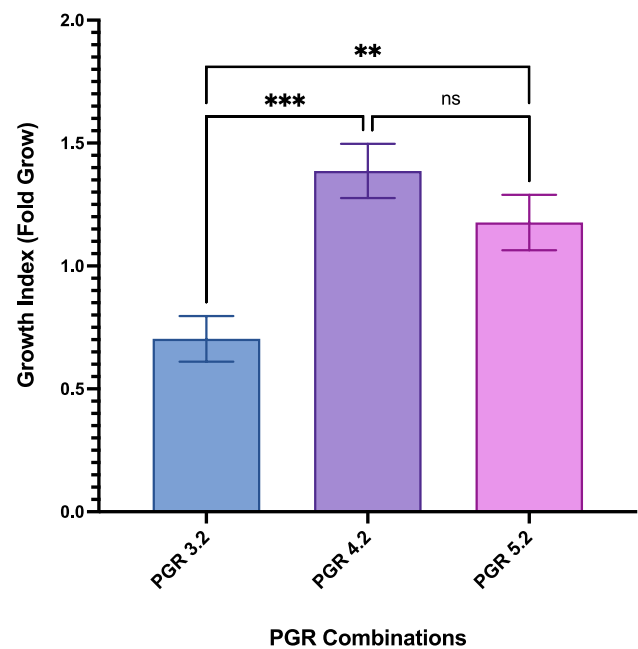


Fig. 3 Effect of PGR 3.2, PGR 4.2 and PGR 5.2 combinations on the proliferation of Sarigelin variety callus tissues (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$, ns: not significant)

Table 4 Morphological information of callus induced by different PGR combinations at the 42nd day of culturing

PGR combination	Callus induction ratio	Adventive explant induction		Callus morphology	Callus color
		Root	Shoot		
PGR 1.1	66.08%	0.00% ^d	0.00% ^c	Friable	Brown > green
PGR 1.2	31.11%	0.00% ^d	0.00% ^c	Compact	Brown > white > green
PGR 2.1	56.63%	0.00% ^d	0.00% ^c	Compact	White > green
PGR 2.2	70.71%	0.00% ^d	0.00% ^c	Compact	White > green
PGR 3.1	77.22%	13.33% ± 5.77 ^c	2.00% ± 1.00 ^b	Friable	Green > yellow
PGR 3.2	93.33%	16.11% ± 3.00 ^{bc}	0.00% ^c	Friable	Green > yellow
PGR 4.1	64.51%	0.00% ^d	0.00% ^c	Compact	Green > white
PGR 4.2	83.33%	0.00% ^d	0.00% ^c	Compact	Green > white
PGR 5.1	61.67%	0.00% ^d	0.00% ^c	Friable	Green > white
PGR 5.2	85.83%	0.00% ^d	0.00% ^c	Friable	Green > yellow > white
PGR 6.1	70.00%	14.33% ± 5.00 ^b	7.33% ± 2.00 ^a	Compact	Brown > green
PGR 6.2	67.77%	53.11% ± 3.00 ^a	0.00% ^c	Compact	Green > white
PGR 7.1	69.36%	0.00% ^d	0.00% ^c	Compact	Green > white
PGR 7.2	65.55%	0.00% ^d	0.00% ^c	Compact	Green > white
PGR 8.1	62.22%	0.00% ^d	0.00% ^c	Compact	White > green
PGR 8.2	74.44%	0.00% ^d	0.00% ^c	Friable	Green > yellow > white

*The difference between the values with the same letter in the adventive explant induction column is not significant according to the DMR test ($p < 0.005$)

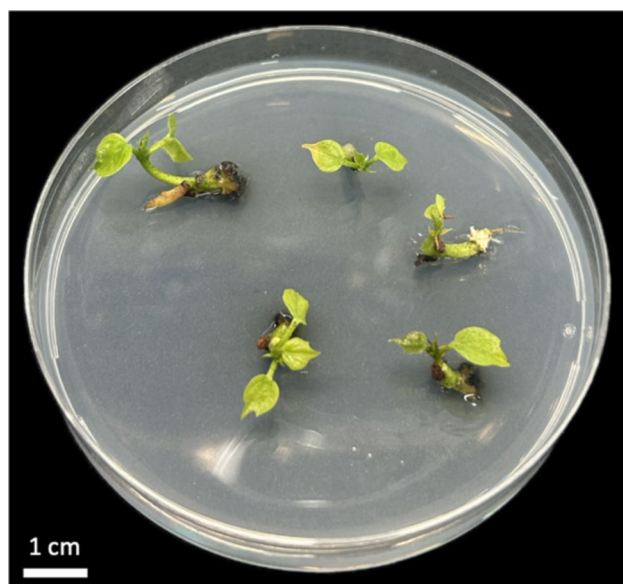


Fig. 4 The stem formation from cotyledonary nodes on MS medium after 21 days of culture

in our study resulted in a higher callus proliferation with 1.387 ± 0.110 -fold growth.

***In vitro* shoot formation**

In this study, cotyledonary nodes of Sarigelin varieties grown in MS medium used for the establishment of *in vitro* propagation systems showed stem regeneration within 21 days. Cotyledonary nodes of Sarigelin varieties showed a 100% stem formation rate in MS medium. After 21 days, the stem formation obtained from the cotyledonary nodes is shown in Fig. 4.

***In vitro* rooting**

After 21 days, the propagated plantlets were transferred to the rooting medium. Significant differences in root length, stem growth, and lateral root development were observed among the different rooting media. After 30 days in the rooting medium, MS medium supplemented with 1.0 mg/L IBA showed a higher rooting rate, root length, and lateral root number than 1/2 MS, MS, and PGR 6.2. IBA not only significantly improved properties related to the root organ such as, rooting rate, root length, and lateral root development, it also significantly affected stem length. Detailed statistics for the medium used in the rooting stage are shown in Table 5.

Table 5 The effect of rooting media on propagated NCC stems

Rooting media	MS medium	PGR 6.2	1/2 MS	MS + 1.0 mg/L IBA
Root induction (%)	26 ± 11	40 ± 00	20 ± 00	73 ± 11
Root length (cm)	5.00 ± 0.5	5.90 ± 0.89	4.50 ± 0.5	8.33 ± 0.57
Lateral root number	6.66 ± 1.15	10.60 ± 1.67	6.0 ± 1.0	22.50 ± 2.88
Stem length (cm)	3.0 ± 0.5	4.33 ± 0.57	2.40 ± 0.36	7.0 ± 1.0

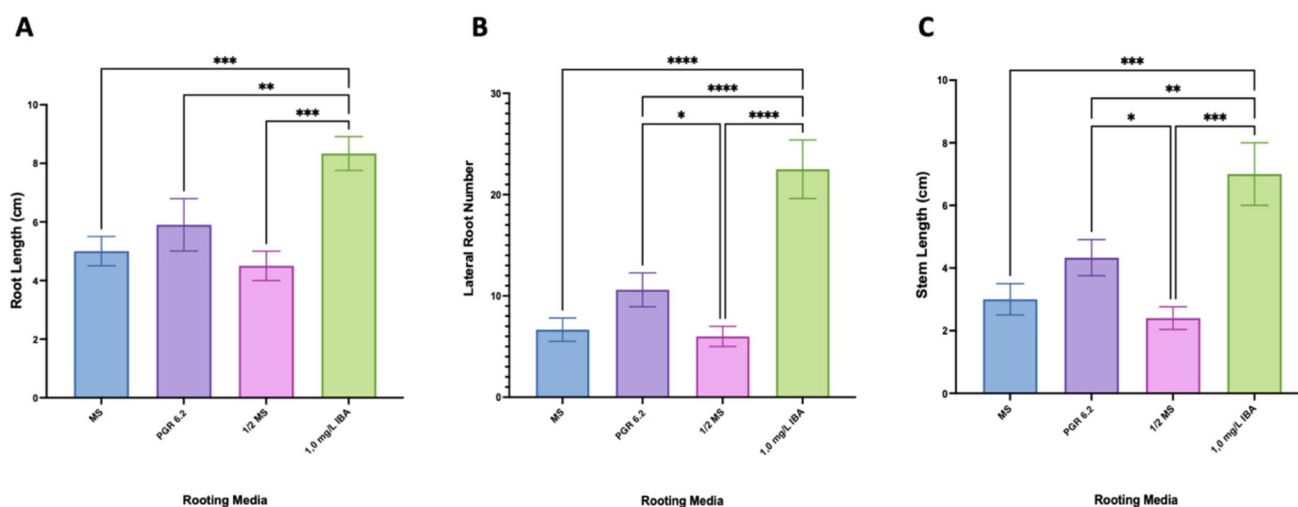


Fig. 5 Effect of different rooting media on *in vitro* grown plant morphology. **A:** Root length, **B:** Lateral root numbers, **C:** Stem length

The significant differences of the media used in the rooting in terms of root length, number of lateral roots and stem length are shown in Fig. 5.

A review published by Arya and Husen (2022) examined the relationship between rooting and IBA in various plants. The article reported that IBA is the most widely used hormone due to its high rooting capacity and emphasized that auxins are critical to initiating the rooting response. IBA, being an auxin derivative, is responsible for root induction. Similarly, using IBA in this study positively affected root development of *in vitro*-grown plantlets of the Sarigelin variety. Using 1.0 mg/L IBA significantly increased root induction, lateral root number, and root length. However, the use of cytokinin in addition to IBA resulted in a significant decrease in these values. Besides the positive effects of IBA on root growth, it also positively affected, and significantly increased stem length compared to other rooting media. When all these results are analyzed, MS medium supplemented with 1.0 mg/L IBA has a significant potential for rooting in micropropagation systems to be established for NCC.

Acclimatization

After rooting, 51-day-old plants were transferred to autoclaved soil containing 10% perlite for acclimatization. Fifteen plants were propagated using the previously mentioned protocol and survived for ten days. Figure 6 shows the representative plant subjected to acclimatization with leaf, stem, and root structure after rooting, and the pot system used for acclimatization.

Effects of PGR combinations on the root suspension cultures

Root tips of 7-days-old Harem II and Sarigelin varieties were grown in liquid MS and PGR 6.2 medium for 21 days. Root tissues were removed from the medium and analyzed statistically. Root tissues obtained from Harem II and Sarigelin variety were cultured in PGR 6.2 because of the high indirect adventitious root organ development rate during callus induction.

Root tissues of the Harem II variety demonstrated 4.143 ± 0.150 -fold growth in MS medium and 2.667 ± 0.452 -fold growth in PGR 6.2. In comparison, root tissues of the Sarigelin variety achieved 6.697 ± 0.620 -fold

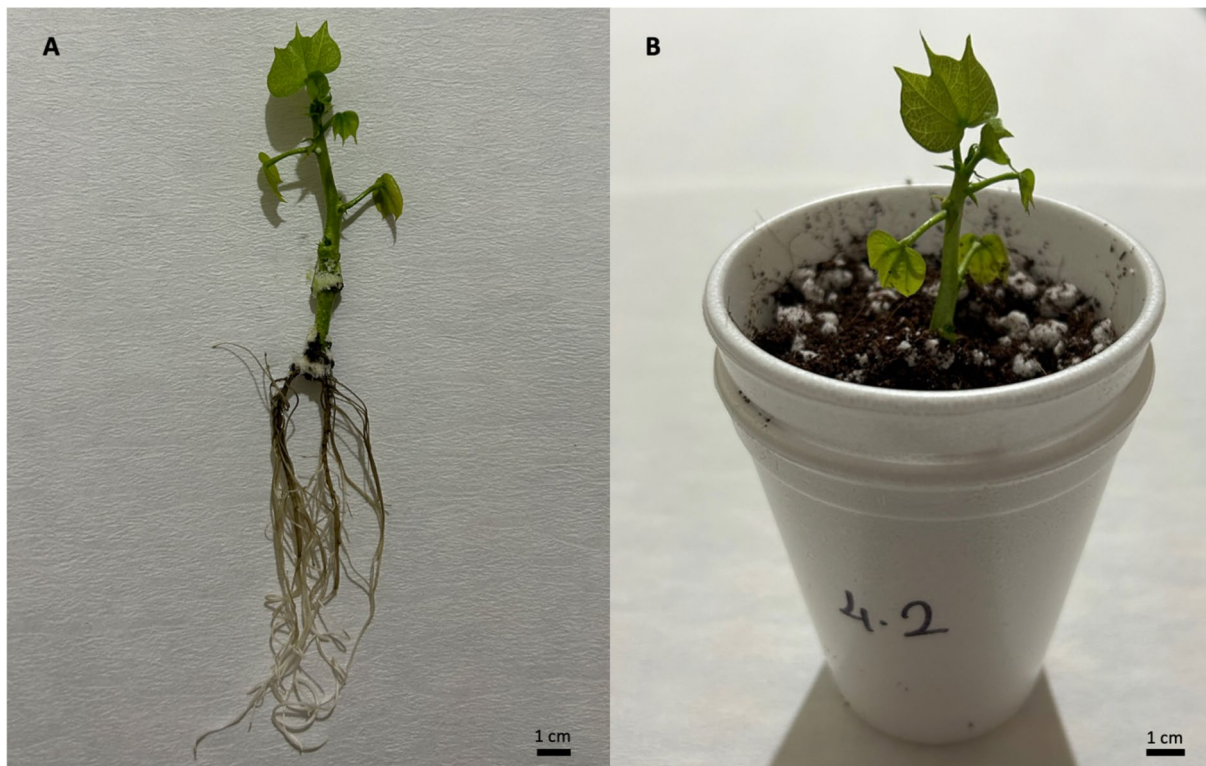


Fig. 6 Acclimatization process for Sarigelin variety. **A:** Representative 51-day-old plant, rooted on MS medium supplemented with 1.0 mg/L IBA **B:** Same plant after 10 days from transfer to soil containing 10% perlite for acclimatization

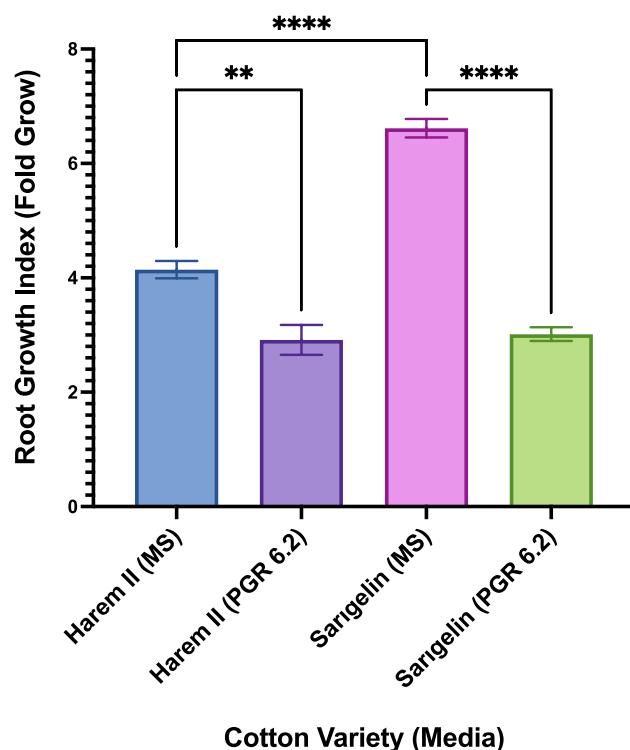


Fig. 7 Distribution of growth indexes of root tissues of Harem II and Sarigelin varieties according to cotton variety and medium after 21 days of culture

growth in MS medium and 3.320 ± 0.535 -fold growth in PGR 6.2.

As a result of the analysis, it was determined that the root tissues of the Sarigelin variety cultured in the MS medium exhibited significantly higher growth compared to those of Harem II ($p < 0.005$). In contrast, both Harem II and Sarigelin root tissues demonstrated significantly higher growth when cultured in the MS medium compared to the PGR 6.2 medium ($p < 0.001$). The growth indices of root tissues cultured under different conditions are presented in Fig. 7.

Increased auxin levels inhibit epidermal cell growth and delay root elongation, resulting in shortened primary roots (Qin et al. 2023). Accordingly, the gradient created using exogenous auxin is significant for primary root growth. Therefore, the concentrations of auxin derivatives used for *in vitro* root growth are critically important (Qin and Huang 2018). Additionally, in a study investigating the effect of auxin and cytokinin balance on root architecture in maize, it was determined that even the use of auxin and cytokinin at much lower concentrations than those in our study, but with the same auxin/cytokinin ratio, had an inhibitory effect on primary root elongation compared to the control (Rivas et al. 2022).

Aligned with this information, in this study, PGR 6.2 containing higher auxin concentration than cytokinins showed a significant inhibitory effect on the growth of both Harem II and Sarigelin root tissues compared to MS medium. In addition, it was found that the root tissues obtained from the Sarigelin variety had a higher growth capacity than those obtained from the Harem II variety.

The root tissues of the Harem II and Sarigelin variety cultured in MS medium and PGR 6.2 differed morphologically. Roots cultured in the MS medium were light-colored, and long lateral roots were observed. Roots cultured in the PGR 6.2 developed darker-colored and shorter lateral roots. The morphology of Harem II and Sarigelin variety root tissues cultured in the MS medium and PGR 6.2 medium are shown in Fig. 8.

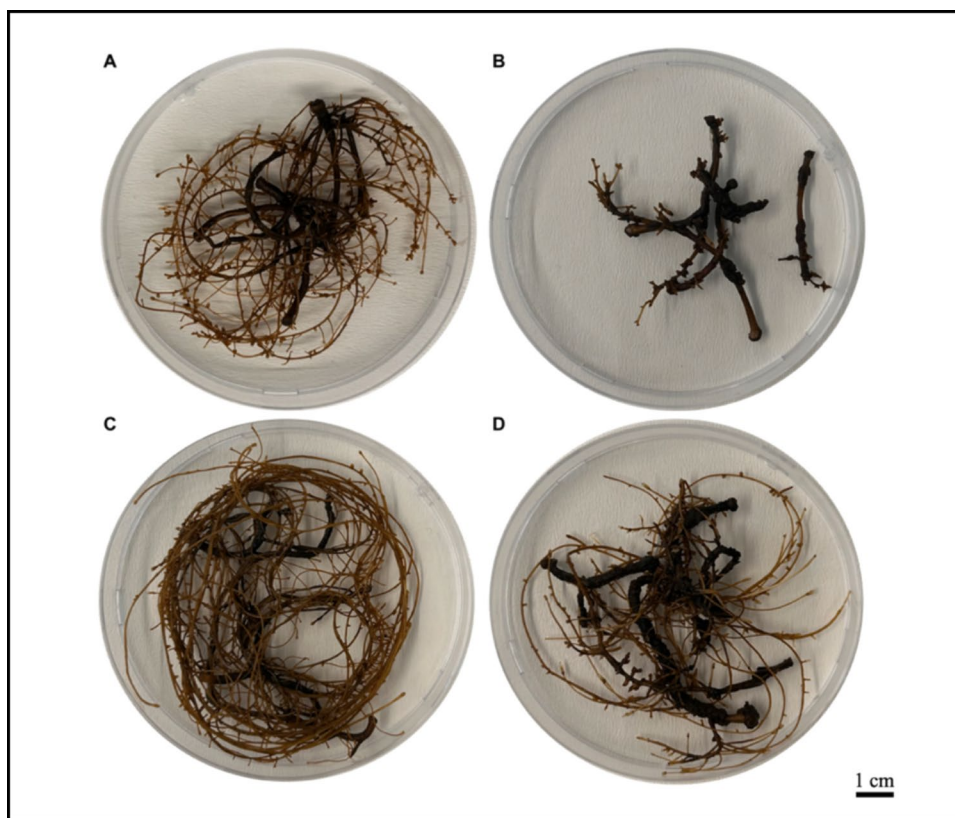
HPLC analyses

In vitro suspension-cultured roots of Harem II variety and Sarigelin variety were used to compare and inspect the effect of genotypic differences on their secondary metabolite profiles by HPLC analysis. The effects of PGR content of the root tissue growth medium on the secondary metabolite profiles were also examined.

Aside from the roots, the secondary metabolite content of callus tissues of Sarigelin variety was also examined. Hence, data regarding the secondary metabolite profiles of different NCC tissues were obtained. For the quantification of secondary metabolites with HPLC, calibration curve was established with at least five different concentrations for each compound. The R^2 values were calculated as > 0.9966 . The secondary metabolite contents of the *in vitro* grown tissues were calculated for each compound using the regression equation obtained from the compound's calibration graph. The retention time, linear range, and correlation coefficient of the analyzed secondary metabolites are given in Supplementary Table 1. Chromatograms for each sample group are presented in Supplementary Fig. 1.

As a result of HPLC analyses, it was determined that the secondary metabolite content in cotton plants was significantly affected by the genotype, tissue source, and the environmental conditions in which the tissue development occurred. The Sarigelin variety was revealed to have significant potential as a production source for the analyzed secondary metabolites, especially catechin, chrysophanol, and physcion. The data related to HPLC analysis are given in Table 6. Ten secondary metabolites were quantified by HPLC analysis of root tissues. Among these, protocatechic acid, ellagic acid, chrysophanol, and physcion were detected for the first time in white cotton Harem II variety *in vitro* root extracts. Additionally, all secondary metabolites analyzed by HPLC were detected for the first time in the NCC Sarigelin *in vitro* root tissues. Both Harem II

Fig. 8 Root tissues obtained from suspension cultures. **A:** MS medium/root tissue of Harem II variety, **B:** PGR 6.2/ root tissue of Harem II variety, **C:** MS medium/ root tissue of Sarigelin variety, **D:** PGR 6.2/ root tissue of Sarigelin variety



and Sarigelin varieties showed remarkable potential compared to the plants reported as suitable sources for these ten secondary metabolites in previous studies (Table 6). Ellagic acid and chrysophanol were found to be significantly higher in the root tissues of Sarigelin cultured in MS medium compared to the root tissues of Harem II ($p < 0.005$). On the other hand, protocatechic acid, catechin, gentisic acid, caffeic acid, rutin, p-coumaric acid, and physcion were found to be significantly higher in the root tissues of the Harem II variety compared to Sarigelin ($p < 0.005$). Only p-coumaric acid was significantly higher in the root tissues of Sarigelin grown in the PGR 6.2 than in the root tissues of the Harem II ($p < 0.005$). Catechin, gentisic acid, p-coumaric acid, coumarin, rutin, chrysophanol, and physcion were significantly higher in Harem II root tissues compared to Sarigelin root tissues ($p < 0.005$).

Moreover, anthraquinone-derived secondary metabolites were found to be more abundant than flavonoid-type secondary metabolites in root tissues of both cotton varieties. Parallel to this result, in a study comparing leaf and root extracts of *Coccoloba mollis* in terms of anthraquinones, these metabolites were found only in root extracts (Barros et al. 2011) and it was previously reported that major anthraquinones were synthesized in the roots of the plant in *Rheum tanguticum*, which has a very important place in traditional Chinese medicine (Li et al. 2023). In the light of this data, it would be more advantageous to use root tissues

in studies targeting anthraquinone biosynthesis and different pathways related to this process on the Sarigelin variety.

Metabolites with remarkably higher quantity in Harem II and Sarigelin varieties differed in conditions where medium type was constant and only the genotypic differences took effect. For the opposite case of same genotype but different medium content, Harem II and Sarigelin varieties were again distinctive regarding the quantity of the metabolites. Therefore, both genotypic differences and PGR contents of the growth medium were determined to be factors significantly affecting the metabolite quantities in the analyzed cotton varieties. Comparative HPLC analysis with ANOVA test results for statistical significance are given in Fig. 9 for Sarigelin and Harem II root tissues cultured in MS and PGR 6.2.

HPLC analysis was also carried out for callus tissues of the NCC Sarigelin variety. NCC callus tissues were induced using three different plant growth regulator combinations, PGR 3.2, PGR 4.2, and PGR 5.2, in order to understand the effect of PGR combinations used in callus induction on the secondary metabolite profile of the callus. The PGR combinations used for the callus tissues significantly differed for seven analyzed metabolites out of the total ten. Protocatechuic acid, catechin, gentisic acid, caffeic acid, and coumarin were significantly higher in callus induced by the PGR 3.2. Conversely, chrysophanol was significantly higher in the PGR 4.2, and physcion in the PGR 5.2-induced callus

Table 6 Comparative HPLC data of different cotton varieties and tissue sources

Metabolite	Harem II root		Sangelin root		Sangelin callus		Other sources	Amount ($\mu\text{g}/\text{mg}$)	References
	MS ($\mu\text{g}/\text{mg}$)	PGR 6.2 ($\mu\text{g}/\text{mg}$)	MS ($\mu\text{g}/\text{mg}$)	PGR 6.2 ($\mu\text{g}/\text{mg}$)	PGR 3.2 ($\mu\text{g}/\text{mg}$)	PGR 4.2 ($\mu\text{g}/\text{mg}$)			
Protocatechuic acid	0.04 $\pm$ 0.00	ND	ND	ND	3.46 $\pm$ 0.04	1.15 $\pm$ 0.13	<i>Malus baccata</i>	1.02	Dadwal et al. (2018)
Catechin	6.9 $\pm$ 0.19	2.59 $\pm$ 0.08	2.0 $\pm$ 0.11	1.57 $\pm$ 0.04	127.9 $\pm$ 0.8	94.28 $\pm$ 0.23	<i>L. sativum</i>	3.39	Rababah et al. (2011)
Gentisic acid	0.7 $\pm$ 0.02	0.05 $\pm$ 0.01	ND	0.77 $\pm$ 0.02	0.63 $\pm$ 0.04	0.05 $\pm$ 0.04	<i>G. hirsutum</i>	0.34	Kouakou et al. (2009)
Caffeic acid	2.0 $\pm$ 0.06	1.03 $\pm$ 0.13	0.08 $\pm$ 0.06	0.93 $\pm$ 0.02	1.52 $\pm$ 0.01	0.21 $\pm$ 0.01	<i>G. hirsutum</i>	1.89	Kouakou et al. (2009)
Rutin	1.6 $\pm$ 0.03	0.34 $\pm$ 0.02	0.38 $\pm$ 0.07	ND	0.34 $\pm$ 0.02	0.26 $\pm$ 0.03	<i>Artemisia carvi-folia</i>	1.39	Khan and Dilshad (2023)
Ellagic acid	1.4 $\pm$ 0.02	2.32 $\pm$ 0.06	3.0 $\pm$ 0.06	2.06 $\pm$ 0.05	ND	ND	<i>Ananas comosus</i>	1.796	Lubaina et al. (2020)
Chrysophanol	53.4 $\pm$ 0.7	92.38 $\pm$ 1.73	86.2 $\pm$ 0.6	64.36 $\pm$ 3.01	1.10 $\pm$ 0.03	1.56 $\pm$ 0.05	<i>Senna occidentalis</i>	20.47	Al-Hamoud et al. (2023)
Physcion	13.0 $\pm$ 1.0	28.11 $\pm$ 1.94	14.1 $\pm$ 0.5	18.02 $\pm$ 0.37	0.18 $\pm$ 0.04	0.72 $\pm$ 0.17	<i>R. japonica</i>	6.71	Nawrot-Hadzick et al. (2018)
<i>p</i> -Coumaric acid	0.57 $\pm$ 0.0	0.15 $\pm$ 0.03	0.4 $\pm$ 0.04	0.42 $\pm$ 0.02	ND	ND	<i>Ananas comosus</i>	0.53	Lubaina et al. (2020)
Coumarin	0.2 $\pm$ 0.02	0.25 $\pm$ 0.01	0.2 $\pm$ 0.01	0.17 $\pm$ 0.03	0.20 $\pm$ 0.03	0.08 $\pm$ 0.02	<i>Melilotus officinalis</i>	1.19	Martino et al. (2006)

ND Not Detected

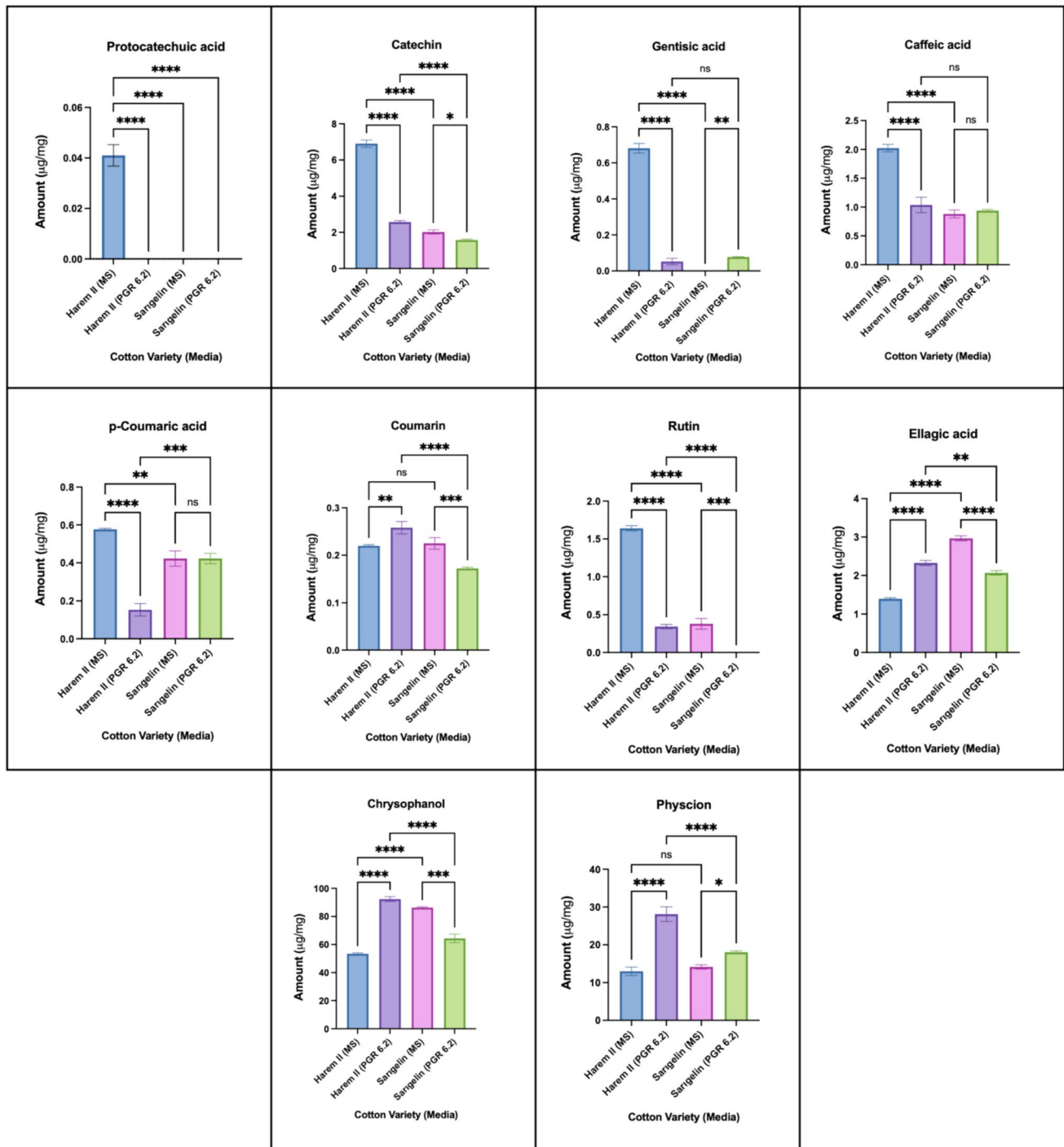


Fig. 9 Comparative HPLC analysis results of root tissues of Sarigelin and Harem II cultured in MS and PGR 6.2

tissues. Comparative HPLC analyses of Sarigelin variety callus tissues induced by different PGR combinations with ANOVA test results for statistical significance are shown in Fig. 10. Considering these data, we revealed that the PGRs used during callus induction from the Sarigelin variety significantly affected the secondary metabolite profiles. It was stated above that anthraquinone-derived secondary

metabolites were found more than flavonoid-derived secondary metabolites in both Sarigelin and Harem II variety root tissues. However, when HPLC analyses were examined, there is not enough evidence for such a generalization for naturally colored cotton callus tissues.

Catechin has significant pharmacological potential and has been isolated from many sources (Ganeshpurkar and

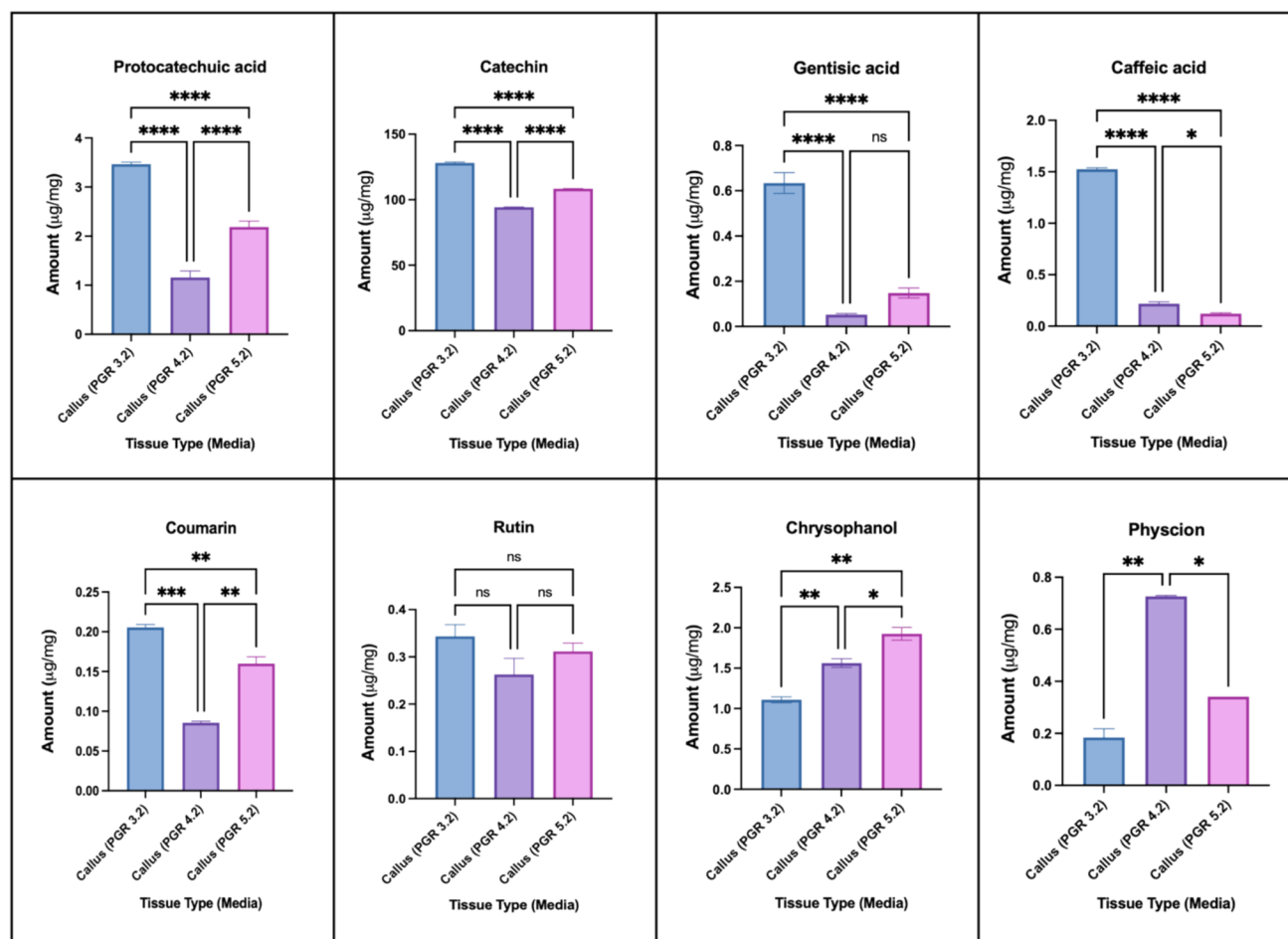


Fig. 10 Comparative HPLC analysis results of Sarigelin variety callus tissues induced in PGR 3.2, PGR 4.2 and PGR 5.2

Saluja 2020). The highest amount of catechin was quantified as 0.980 µg/mg in a study conducted on the skin of white and red grapes, which are characterized as rich in flavonoids (Hrnčič et al. 2019). However, in this study, the callus of the Sarigelin variety induced by the PGR 3.2 combination contained 127.983 µg/mg catechin. Therefore, NCC callus has a major potential to be a rich source of catechin.

Comparison of secondary metabolite profiles of root and callus tissues

Throughout this study, root and callus tissues of the NCC Sarigelin variety were induced and grown in different media. At this point, comparing secondary metabolic profiles of root and callus tissues allowed us to observe the effect of tissue type on the analyzed metabolite contents. Comparative HPLC analyses of Sarigelin variety root and callus tissues are shown in Fig. 11.

According to comparative HPLC analyses, callus tissues of the Sarigelin variety contained significantly more protocatechuic acid, catechin, gentisic acid, and caffeic acid

than root tissues. In the context of coumarin and rutin, no significant difference was observed between the callus and root tissues. However, roots of Sarigelin contained significantly more p-coumaric acid, ellagic acid, chrysophanol, and physcion than callus tissues. Therefore, it is understood that different tissues and organs in NCC result in significant differences of secondary metabolite profiles.

One of the findings supporting the fact that different tissues contain significant differences in terms of secondary metabolite derivatives is the catechin amounts in the root and callus tissues of Sarigelin variety. As a result of HPLC analysis, it was found that the callus tissues of the Sarigelin variety contained dramatically more catechins than the root tissues of the Sarigelin variety. Similar results were obtained in a study by Liu et al. (2009) in which tissue-specific catechin accumulation was investigated in *Camellia sinensis* plants. In HPLC analyses performed throughout the study, catechin accumulation was detected in callus tissue, while no catechin accumulation was observed in root tissue.

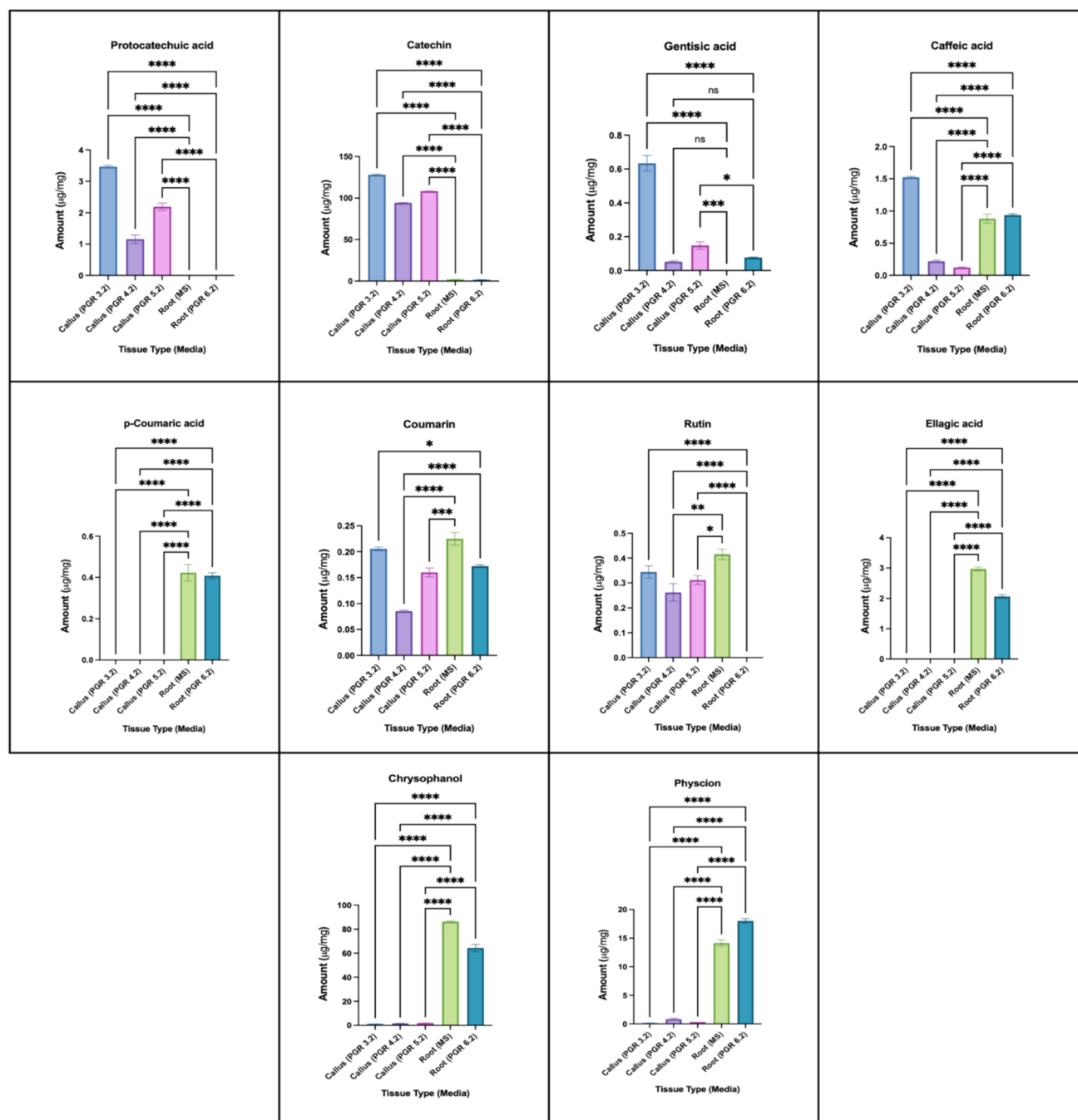


Fig. 11 Comparative HPLC analyses of Sarigelin variety root and callus tissues

These results provided important findings regarding the secondary metabolite profiles of NCC callus and root tissues. These findings make NCC an important candidate plant for the production and biotechnological utilization of catechin flavonoid, which is frequently used in cancer research, or anthraquinone-type secondary metabolites, which can be used both as natural pigments and have pharmaceutical potential.

Conclusion

The increasing awareness of the global climate crisis and the subsequent sustainability goals of the European Union have led manufacturers in the textile industry, as in many other industries, to search for new raw material alternatives. Although NCCs are one of the most critical and eco-friendly alternatives for the textile industry, their use has yet to become widespread due to their inadequacies

in color scale and production efficiency. Biotechnological studies are required to ameliorate these adverse effects. However, more research on optimizations is needed for the rapid and stable production of NCC tissues which can be used as experimental material in biotechnology.

In this study, plant tissue culture procedures, one of the fundamental steps for biotechnological research on plants, were applied and optimized for NCC, while analysis of secondary metabolite profiles was also carried out. As a result of our study, we obtained significant data for multiple PGR combinations to be used for callus induction, suitable methods and media for *in vitro* propagation, establishment of root suspension culture, and factors affecting root growth. In addition, HPLC analyses provided insightful data on the secondary metabolite profiles of NCC and white cotton. For many secondary metabolites, white cotton and NCC root and callus tissues were evaluated for the first time in our study.

Using the tissue culture methods and PGR combinations tested in this study it will be possible to obtain various NCC tissues rapidly *in vitro*, paving the way for biotechnological studies that can counter the negative industrial properties of NCC. As a result of HPLC analyses, NCC is determined to be rich in secondary metabolites with high commercial value, especially catechin, chrysaphanol, and physcion. Notable data have been obtained for the large-scale production of secondary metabolites suitable for use in food, cosmetics, health, and textile industries using NCC. In this context, our findings have the potential to be a pioneering study for the textile industry and NCC, to contribute novel strategies of tissue culture to the scientific literature, and to serve as a groundwork for further studies.

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Author contributions Onur Aydın: Investigation, Methodology, Writing original draft, Writing – review & editing Neslihan Turgut Kara: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Data availability All data supporting the findings of this study are available within the paper and its Supplementary Information.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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