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Comparative Analysis of Protoplast Isolation and Somatic Embryogenesis from Sweetpotato Meristems

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Abstract
This study evaluated fifteen sweetpotato genotypes for mesophyll protoplast isolation and regeneration from *in vitro* plants. The initial cell division was observed on MS medium supplemented with plant growth regulators. Another experiment assessed the regenerative capacity of the apical meristem and axillary buds for embryogenic and organogenic callus formation following *in vitro* shoot regeneration, using auxins and cytokinins. Results showed that two plant growth regulators, at 2 mg L^{-1} 2,4-D or 2,4,5-T, produced a very high percentage (100%) of both callus types across all genotypes. Histological analyses confirmed these findings. For shoot induction, zeatin and kinetin (both at 2 mg L^{-1}) were tested, resulting in shoot formation in only a few genotypes. Fully regenerated plants were obtained in one genotype, LI1, on MS medium with kinetin. A long-term yellow-orange callus, which did not show signs of regeneration, was examined for plant pigment composition.

Keywords Sweetpotato, Protoplasts, Tissue cultures, In vitro regeneration, Plant growth regulators, Histology, Plant pigments

Introduction
Sweetpotato (*Ipomoea batatas* L. Lam.) is a sweet-tasting root vegetable with flesh that ranges from white and creamy to orange and purple [1]. Typically high in starch, fiber, vitamins, and antioxidants, it originates in tropical South America [2]. Currently, China leads sweetpotato production, with 51 million tons in 2024, according to FAO statistics [3]. In Europe, the main producing countries are Spain, Italy, and Portugal [4], with limited or no cultivation in the Nordic countries [5]. Today, sweetpotato is considered a superfood [6] and provides nutritional benefits for human health [7].
Sweetpotato has been a focus of plant research for over four decades, with early reports documenting in vitro regeneration methods from various tissues to produce new plants via direct organogenesis or somatic embryogenesis [8-15]. These studies evaluated and compared plant growth regulators (PGRs) for initiating regeneration and improving embryogenesis-based protocols. Various explants, including lamina and petiole [16], node [17], apical meristem [18], axillary shoot [19], shoot tip [20], and adventitious root [21], have been used to induce regeneration in sweetpotato.

Liquid cultures have been used for both non-embryogenic and embryogenic callus cultures of sweetpotato [22] and for suspension cultures [23] to support induction, growth, and production of plant pigments and phenolic compounds [24]. A protocol for virus-free *in vitro* cultures was developed and widely adopted [25].

Today, sweetpotato *in vitro* cultures are used to multiply planting material for farmers [26] rapidly and to provide long-term storage or preservation of different lines in Africa [27]. The publication by Doyina et al. [28] provides a comprehensive overview of the essential role of plant growth regulators in sweetpotato micropropagation, and the review by Bahera et al. [29] highlights the importance of various tissue culture techniques for rapidly multiplying elite, virus-free planting material.

All this accumulated experimental knowledge of sweetpotato in vitro culture provides a strong foundation for developing successful protocols using more advanced techniques, such as genetic transformation [30-35] or recently developed gene-editing protocols reviewed by Lyu et al. [36].

Many of these techniques rely on successful protoplast cultures that regenerate transformed or gene-edited plants. For these experiments, a reliable, consistent plant regeneration protocol is essential. Success often depends on the selected sweetpotato cultivars and the combination of plant growth regulators used throughout the process [37-40].

Therefore, our study aimed to screen a wide range of sweetpotato germplasm from the CIP (International Potato Center in Lima, Peru) breeding program to identify the most responsive genotypes for protoplast isolation and regeneration via somatic embryogenesis or organogenesis, using various plant growth regulators, to support future experimentation and use in new plant breeding technologies

Results

Sweetpotato Protoplast Cultures

Three-week-old sweetpotato leaves from 14 CIP genotypes and the commercial variety Orleans, grown in vitro, were collected in a 9 cm Petri dish containing 0.5 M sorbitol plasmolytic solution for 30-35 minutes (Figure 1a, b). The solution was replaced twice with fresh solution, and the leaves were cut into small pieces with a scalpel or scissors. The plasmolytic solution was then replaced with an enzymatic mixture containing 1.5% (w/v) Cellulase R10, 0.75% (w/v) Macerozyme R10, 0.6 M D-Mannitol, 10 mM MES monohydrate, 10 mM $CaCl_2.2H_2O$, and 0.1% (w/v) bovine serum albumin (Sigma-Aldrich). Enzymatic digestion was carried out for 6 hours at 25°C. However, this duration was insufficient to digest the leaf tissue of all tested genotypes, including the early-maturing line EM 5 (Figure 1e), and only a few protoplasts were released into the solution. Therefore, the incubation was extended to 16 hours overnight (Figure 1f). This adjustment resulted in a high yield of isolated protoplasts (about 3.106) on average after washing and centrifugation (Figure 1c) in this genotype. The washing and centrifugation steps were performed as described in [41]. In some sweetpotato genotypes, small tissue debris remained in the protoplast pellet after washing, so an additional 0.43 M sucrose gradient was used to purify the samples (Figure 1d).

Freshly isolated sweetpotato protoplasts were further cultured at 25 °C in the dark, either in liquid or solid medium (same composition), with the solid medium solidified with 2.8% (w/v) sodium alginate. We tested a wide range of modified cultivation media supplemented with different concentrations and combinations of plant growth regulators. However, only the liquid Murashige and Skoog (MS) [42] medium, ½ MS with macro- and microelements supplemented with MS

vitamins, 0.05 mg L-1 2,4-D (2,4-dichlorophenoxyacetic acid), 0,5 mg L-1 kinetin, 500 mg L-1 casein hydrolysate, 6 mM $CaCl_2 \cdot 2H_2O$, 10 mM L-arginine, 1% (w/v) sucrose, and 0.6 M D-mannitol induced cell wall resynthesis in two CIP genotypes (HI1 and EM5) and initial cell division, as shown in Figure 1h (arrow indicating the first cell division), but only in the EM5 culture. Unfortunately, this culture failed to progress further and did not form microcalli. The other genotype tested in this study showed no cell division after 3 weeks of protoplast cultivation, despite several modifications to the culture medium (data not shown).

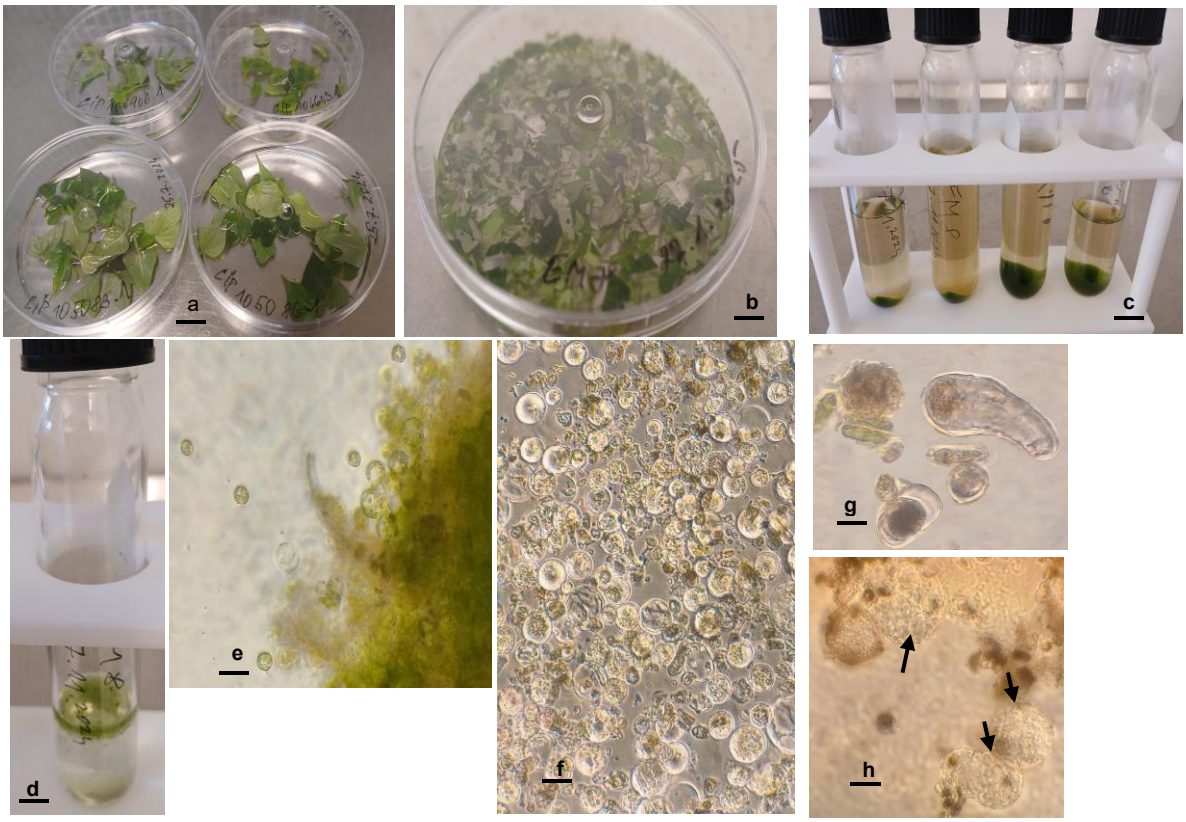


Figure 1. Sweetpotato protoplast isolation and cultivation. (a) Leaves from different in vitro plants originating from CIP, (b) genotype early maturing number 8 (EM8) undergoing overnight enzymatic digestion, (c) freshly isolated protoplasts in a pellet after washing and centrifugation, (d) sucrose gradient used for protoplast purification, (e) undigested leaf tissue after 6 hours of enzymatic treatment genotype early maturing genotype EM5, (f) overnight enzymatic digestion (16 hours) of genotype hight iron 1 HI1, (g) early maturing genotype ME5 in liquid MS medium five weeks after protoplast isolation, (h) initial cell divisions (indicated by arrows) in liquid MS medium; early maturing genotype EM5.

Shoot Meristems and Immature Embryos: Callus Formation, Shoot Induction, and Plant Regeneration

In our experiment, we used shoot meristems from three-week-old *in vitro* plants grown on MS hormone-free medium. All details of these subcultures are schematically shown in **Figure 2.**

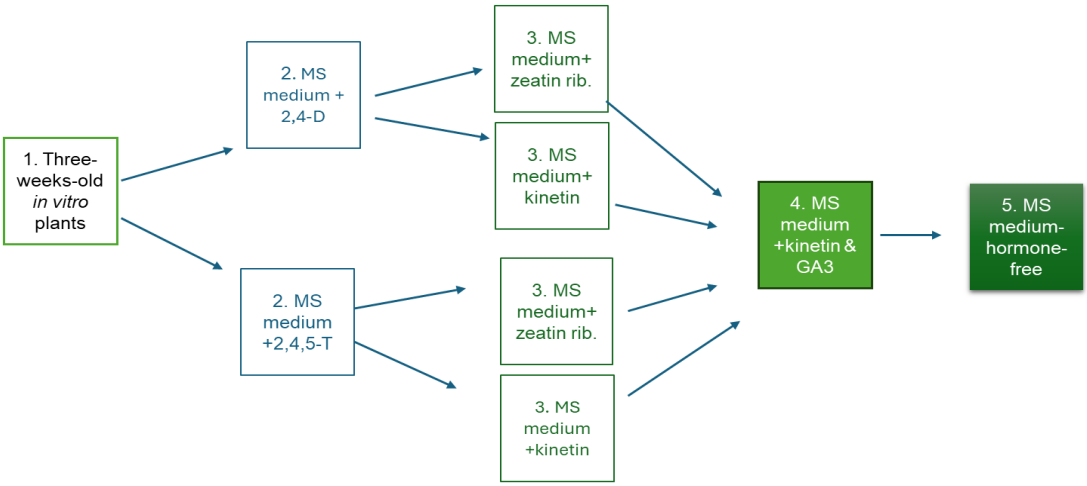


Figure 2. Graphic showing the media used to induce 15 sweetpotato genotypes from calli and shoots derived from apical meristems, axillary buds, and immature embryos.

Aseptically collected apical meristems and axillary buds were transferred to media containing auxins for callus formation (**Figure 3a, b**). The induced calli were then moved to MS medium with cytokinin for shoot induction, followed by media for shoot elongation (Figure 3c-i) and rooting (Figure 3j-l).

Different types of calli were induced in the tested genotypes (Figure 3e-i), and this variation was confirmed through histological analysis of these cultures (as shown in Figures 6b and 6d). The callus developed from the EM2 genotype (Figure 3g) exhibited a deep orange color and was used for plant pigment analysis.



Figure 3. *In vitro* cultures of sweetpotato lines, (a) 15 lines from CIP and Orleans initial cultures, (b) *in vitro* line multiplication for different experiments (CIP 199062.1) EM 8, (c, d) embryogenic callus formation LI 1 on medium MS supplemented with 2,4-D (yellow arrow) and non-embryogenic callus induction (red arrow), (e) early maturing genotype EM 8 with embryogenic callus induced on MS medium supplemented with 2,4,5-T, (f) organogenic callus induces on 2,4-D following transferred to MS -kinetin, early maturing genotype EM2 having an orange colour, (g)) organogenic callus induces on 2,4-D following transferred to MS+kinetin, early maturing genotype EM5 with orang colour, on MS kinetin +GA_3, (h) organogenic callus induces on 2,4-D following transferred shoots induction medium MS -zeatin, early maturing genotype EM8 with dark green colour on MS medium+kinetin. (i) Organogenic callus LI1 induced on 2,4-D, transferred to MS-kinetin; following transfer to MS-kinetin+GA3 in the shoot elongation stage, (j) embryogenic callus formation LI1 on medium MS supplemented with 2,4-D, shoot induction on medium MS+kinetin, (3k, l) shoots and plant formation of LI1 on medium MS+kinetin, following elongation and rooting on MS-hormone-free medium.

Callus culture of genotype EM 8 was induced to form organogenic, dark-green tissue on 2,4-D (Figure 3h). The subsequently transferred shoots, induced on MS medium supplemented with zeatin and later with kinetin, did not produce any shoots. This callus has been used for plant pigment analyses.

In vitro cultures of the LI1 genotype were initially induced on MS medium supplemented with 2,4-D, resulting in organogenic callus formation (Figure 3i and j). However, unlike genotype EM 8, these cultures, after transfer to MS medium supplemented with kinetin for shoot induction, produced shoots (Figure 3k) within 3 weeks. Figure 3k shows the original shoots, which were subcultured and continued to grow on hormone-free MS medium.

Callus formation was analyzed using a generalized linear model (GLM) with a binomial distribution and a logit link. The response variable was the number of calli formed relative to the total number of apical meristems and axillary buds per experimental unit. Sweetpotato genotype, meristem type (apical or axillary), and culture medium composition (growth regulators added to the culture medium) were included as fixed effects. Unbalanced experimental designs were handled due to variability in the number of buds evaluated across treatments.

Estimated probabilities of callus induction were calculated from the estimated margins and expressed as percentages. Multiple comparisons among genotypes within each combination of meristem type and culture media were performed using Tukey's adjustment at $\alpha = 0.05$. Results are presented as the percentage of calli induced, with significance letters indicating no statistically significant differences when letters are identical. All statistical analyses and visualizations were conducted in R (version 4.5.2), an open-source statistical computing software.

As shown in Table 1, callus formation was high across all evaluated sweetpotato genotypes, regardless of meristem type or the auxin used for callus induction, such as MS medium supplemented with 2 mg L-1 2,4-D or 2,4,5-T. No significant differences were observed in callus induction rates among genotypes within each treatment or overall ($p \geq 0.05$). Generally, callus induction rates reached 95%, except for the clones EM3 and LI1 in both media tested and for both explants, indicating a uniform response. The observed numerical differences were not sufficiently consistent to be detected as significant under a conservative statistical framework.

Table 1. Percentage of calli induced in sweetpotato genotypes across shoot meristem types and culture media.

Genotype	Apical meristem 2,4,5-T	Axillary buds 2,4,5-T	Apical meristem 2,4-D	Axillary buds 2,4-D
EM1	100[a]	100[a]	100[a]	100[a]
EM2	95.2[a]	95.0[a]	99.4[a]	99.4[a]
EM3	100[a]	100[a]	100[a]	100[a]
EM4	100[a]	100[a]	100[a]	100[a]
EM5	100[a]	100[a]	100[a]	100[a]
EM6	100[a]	100[a]	100[a]	100[a]
EM7	100[a]	100[a]	100[a]	100[a]
EM8	100[a]	100[a]	100[a]	100[a]
HI1	96.8[a]	96.7[a]	99.6[a]	99.6[a]
HI2	100[a]	100[a]	100[a]	100[a]
HI3	54.9[a]	53.8[a]	91.0[a]	90.6[a]
HI4	100[a]	100[a]	100[a]	100[a]
LI1	62.2[a]	61.2[a]	93.2[a]	92.9[a]
LI2	100[a]	100[a]	100[a]	100[a]
Orleans	100[a]	100[a]	100[a]	100[a]

Shoot formation from cultured calli showed consistent differences across sweetpotato varieties, explant types, and hormonal treatments (Figure 4). Model-adjusted shoot formation rates per callus, estimated using Poisson generalized linear models with correction for the number of cultivated calli, revealed clear patterns both at the genotypic level and in response to plant growth hormones used.

For the apical meristem, shoot formation rates per callus were generally higher under the 2,4,5-T + kinetin and 2,4,5-T + zeatin treatments than under the 2,4-D + kinetin and 2,4,5-D + zeatin treatments. In the 2,4,5-T + kinetin treatment, varieties HI 2 and Orleans showed the highest shoot formation rates, exceeding 9 shoots per callus, and were classified as the highest statistically significant group based on Tukey's test ($\alpha = 0.05$). In contrast, EM4 consistently exhibited the lowest shoot formation rates across all apical meristem treatments, with values near or below 1 shoot per callus, forming a distinct lower-significance group.

A similar pattern was observed with the 2,4,5-T + zeatin treatment, although shoot formation rates were slightly lower than those with 2,4,5-T + kinetin. Once again, HI2 and Orleans showed the highest estimated rates of shoot induction per callus, while EM1, LI1, and EM7 displayed intermediate responses.

Treatments with 2,4-D (2,4-D + kinetin and 2,4-D + zeatin) resulted in lower overall shoot formation rates. However, the relative ranking among varieties remained largely unchanged, with HI2 and Orleans showing the highest shoot induction capacity and EM4 the lowest.

For axillary explants, shoot formation rates per callus were generally lower than those observed in apical explants under the same hormonal treatments. However, the varietal response pattern remained consistent. Under both 2,4,5-T + kinetin and 2,4,5-T + zeatin, varieties HI2 and Orleans again showed the highest shoot induction rates, reaching approximately 5 to 8 shoots per callus, and were classified in the highest significance groups. In contrast, EM4 and EM1 consistently exhibited the lowest shoot formation rates across all axillary explant treatments. Genotype EM2 failed to induce any shoots, producing only yellow-orange proliferating callus masses (Figure 3f).

As observed for the apical meristem, growing on MS medium supplemented with 2,4-D (including 2,4-D+kinetin, 2,4-D+zeatin) resulted in lower shoot induction rates when axillary buds were used as initial explants.

Despite this overall decrease, the relative differences among varieties remained consistent, with the better-performing genotypes.

Under treatments involving 2,4,5-T, shoot formation rates are also higher on 2,4-D-based media, although at lower overall levels (Figure 3i, j).

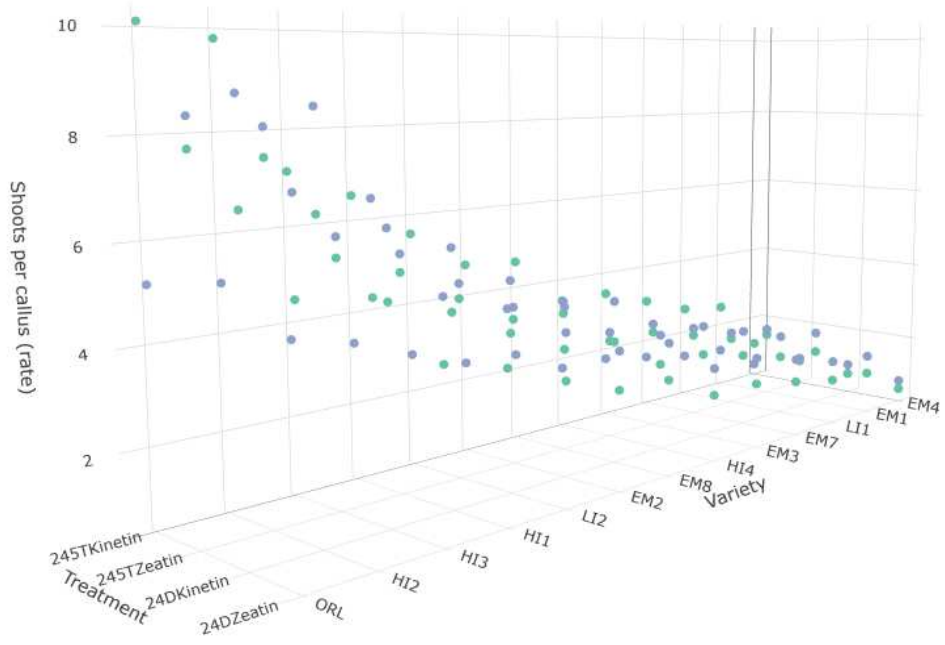


Figure 4. A three-dimensional scatter plot illustrating the model-adjusted shoot formation rates per callus for different sweetpotato varieties under various hormonal treatments and explant types. Each point represents an estimated shoot formation rate from a Poisson generalized linear model. The x-axis indicates sweetpotato varieties, the y-axis shows hormonal treatments, and the z-axis displays the estimated number of shoots per callus. Point color differentiates explant type (apical meristem green color versus axillary buds in blue color).

Callus induction from immature embryos of two sweetpotato genotypes, LI 1 and ME 3, on MS medium supplemented with 2 mg L^{-1} of 2,4-D and 2,4,5-T, respectively, produced only organogenic callus, as shown in **Figures 5g and 5 h**. In long-term culture on MS medium with kinetin alone or on shoot elongation medium with a combination of cytokinin, kinetin, and GA_3, only roots or small shoots formed, respectively. Surprisingly, we have never observed any embryonic-like structures in these two cultures.



Figure 5. Flowering sweetpotato plants in a Biotron. (a) Flower and capsule genotype, low iron 1 LI1 genotype; (b) Early maturing genotype EM3 flowering plant in a Biotron; (c and d) Flower pots with LI1; (e) Immature embryo of LI1 on MS callus induction medium supplemented with 2,4,5-T; (f) Immature embryo of early maturing genotype EM3 on MS callus induction medium supplemented with 2,4,5-T; (g) Organogenic callus of early maturing genotype EM3 induced on MS-2,3,5-T medium, transferred to MS with kinetin; (h) Callus transferred to MS with kinetin and GA_3, where shoots begin to elongate.

Histological Analysis of Induced Calli Cultures

Callus cultures from seven genotypes, including (a) LI 1 on 2,4-D, (b) HI 1, (c) LI2, (d) EM2, (e) EM3 on 2,4-D, (f) LI 2, and (g) HI2 on 2,4,5-T, were initiated from axillary buds of *in vitro* plants on MS medium supplemented with two PGRs. These cultures were grown at 23°C in darkness, then fixed, embedded, sectioned, and stained according to the protocol described by [43, 44]. Histological analysis of the induced callus cultures from axillary buds revealed distinct morphologies, typical of embryogenic or organogenic callus, depending on the genotype and auxin used in the induction medium (see **Figure 6 for details**). Results show that both auxins tested for callus induction can produce embryogenic structures (see Figure 6 for 2,4-D a, b, c, and e; and for 2,4,5-T f and g).

This result also aligns with the visual appearance of the callus cultures, such as callus induced by 2, 4-D (Figure 6a), which clearly shows the early stages of a globular embryogenic structure and matches the appearance of these calli in the culture medium, as shown in Figures 2c and 2d.

However, not all genotypes (e.g., LI 2 and EM 2) formed embryogenic callus on medium supplemented with 2,4-D, as shown in Figures 6b and 6d; these callus cultures produced undifferentiated, even non-organogenic, callus (Figure 2g) during long-term cultivation.

Histological observations of LI 1 callus developed on medium with 2,4-D confirmed the presence of embryogenic cells characterized by very dense cytoplasm, numerous small vacuoles, and a large, round nucleus. The globular-shaped embryos with a visible layer of protoderm cells were also noted (Figure 6a, b). HI1 callus formed on medium with 2,4-D consisted of thin-walled parenchymatous cells, some of which were loosely arranged (Fig. 6c). These cells varied in size, with large vacuoles and irregularly shaped nuclei located at the periphery, containing different numbers of nucleoli (Fig. 6c).

Histological analysis of LI 2 callus grown on medium with 2,4-D revealed its heterogeneous nature, with proembryogenic complexes present. As a result, several cell types could be distinguished (Figure 6d, e). Embryogenic cells with multiple small vacuoles and prominent, round nuclei with one, two, or three nucleoli, along with globular embryo-like structures, were observed (Figure 6d). Additionally, meristematic cells with several visible vacuoles and round nuclei containing one, two, or three nucleoli were also present (Figure 6e).

The EM2 callus formed on medium with 2,4-D was primarily composed of parenchymatous cells with large vacuoles, a peripherally located, irregularly shaped nucleus with one nucleolus, and surrounded by phenolic compounds (Figure 6f). Histological examination of EM3 callus grown on medium with 2,4-D identified several cell types, including meristematic cells, regions of parenchymatous cells, and globular embryo-like structures (Figure 6g).

LI2 callus cultured on medium with 2,4,5-T showed abundant, concentrically arranged meristematic cells with nuclei containing one or two nucleoli, surrounded by parenchymatous cells (Figure 6h). HI2 callus grown on medium with 2,4,5-T was mainly composed of meristematic cells encircled by parenchymatous cells (Figure 6i).

Globular and early heart-shaped embryos with visible suspensor-like structures were also observed (Figure 6i).

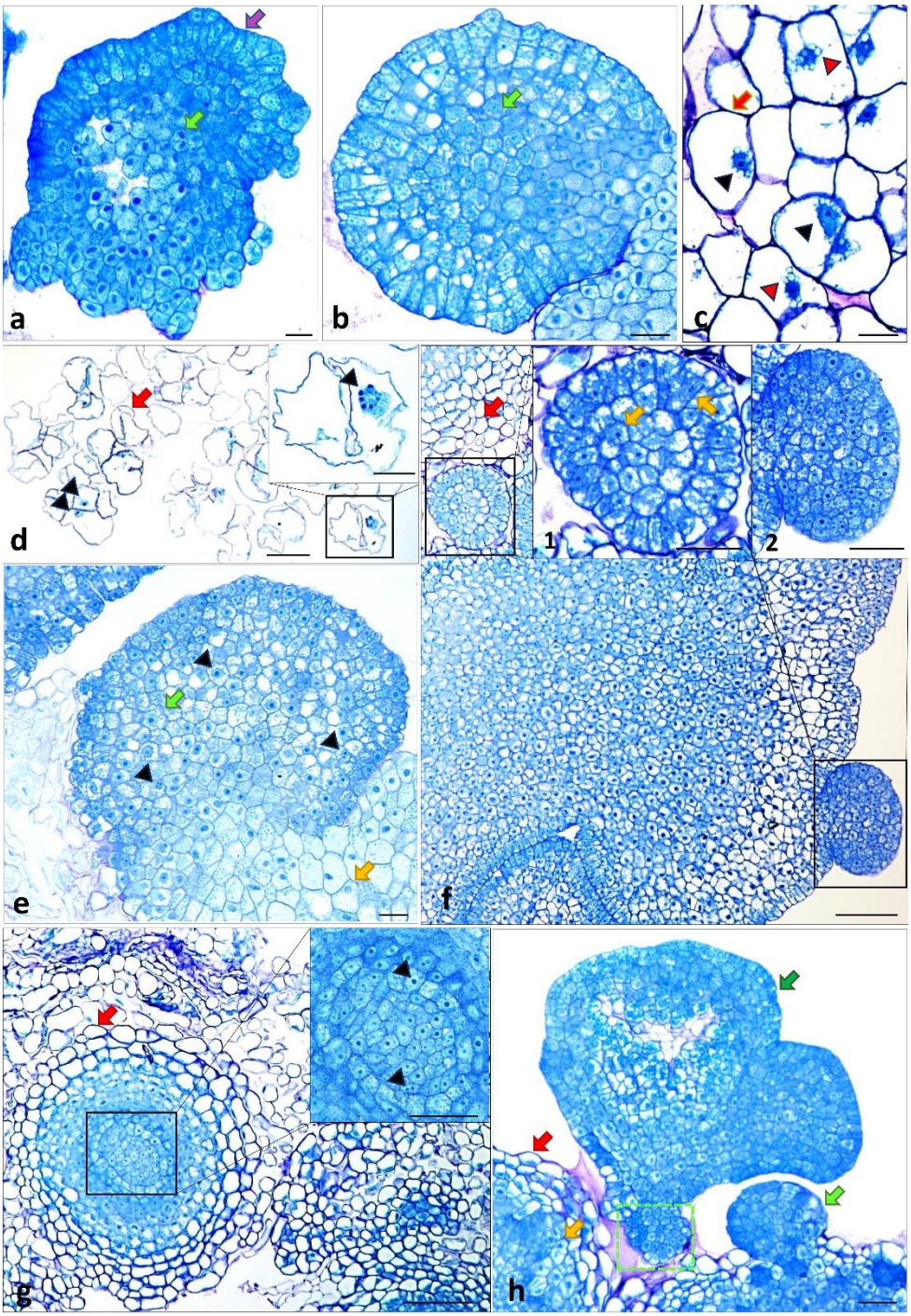


Figure 6. Histological sections of callus induced on MS medium supplemented with 2,4-D or 2,4,5-T. (a, b) LI 1 on 2,4-D, (c) EM 2 on 2,4-D, (d) HI 1 on 2,4-D,(e) LI 2 on 2,4-D, (f) EM 3 on 2,4-D, (g) LI 2 on 2,4,5-T, and (h) Hi 2 on 2,4,5-T. (a) Embryogenic cells with very dense cytoplasm, several small vacuoles, and a large, round nucleus (green arrow) with a visible layer of protoderm cells (purple arrow); (b) Globular-shaped embryo with visible embryogenic cells (green arrow); (c) Parenchymatous cells (red arrow) with a large vacuole, a peripherally located, irregularly shaped nucleus with one nucleolus (black arrowheads), surrounded by phenolic compounds (red

arrowheads); (d) Parenchymatous cells (red arrow) with a large vacuole, an irregularly shaped nucleus with varying numbers of nucleoli (one, two black arrowheads or five inset, black arrowhead); (e) Globular-shaped embryo with visible embryogenic cells containing several small vacuoles (green arrow) and a prominent, round nucleus with one, two, or three nucleoli (black arrowheads), meristematic cells with several vacuoles and round nuclei (yellow arrow); (f) Meristematic cells with several vacuoles and a round nucleus with one or two nucleoli (yellow arrows, inset 1), regions of parenchymatous cells (red arrow), and a globular embryo-like structure (inset 2); (g) Concentrically organized meristematic cells with a nucleus containing one or two nucleoli (inset, black arrowheads), surrounded by parenchymatous cells (red arrow); (h) Meristematic cells (yellow arrow) surrounded by parenchymatous cells (red arrow), with a globular (light green arrow) and early-heart-shaped embryo (dark green arrow), along with a suspensor-like structure (green dashed-line rectangle). Scale bars: 10 µm (e, inset: g); 20 µm (a, b, c; insets: d, f); 50 µm (f, g, h); 200 µm (d).

Analyses of Plant Pigments

FTIR Analyses

FTIR analysis was conducted to detect chlorophylls and carotenoids during the callus induction and growth stages of sweetpotato, using leaves from in vitro-grown corresponding genotypes as controls. As the callus never produced shoots and displayed distinct coloration and morphological characteristics, it may represent a promising plant-based system for secondary metabolite production. Therefore, a preliminary FTIR screening was performed to determine whether the callus contained secondary metabolites. Results are presented in **Figure 7.**

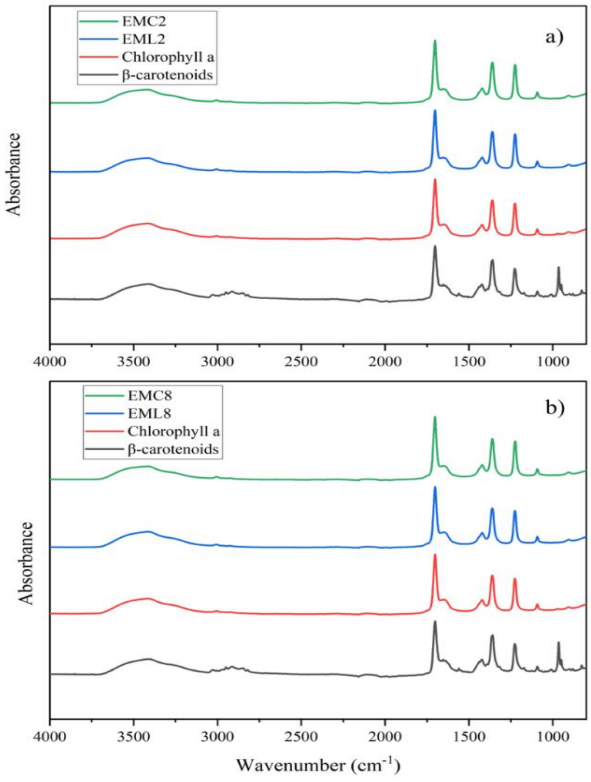


Figure 7. ATR-FTIR spectra of sweetpotato leaf (EML) and callus (EMC) acetone extracts compared to standards. (a) EMC2 (green) and EML2 (blue) overlaid with β-carotene (black) and chlorophyll *a* (red) standards. (b) EMC8 (green) and EML8 (blue) overlaid with standards.

The FTIR spectra show eight prominent absorption peaks. A broad, medium-strength band appears between 3700 and 3065 cm^{-1}, with two shoulders at 3542 cm^{-1} and 3268 cm^{-1}. A small, low-intensity peak is observed at 3004 cm^{-1}. A sharp peak occurs around 1701 cm^{-1}, followed by a lower-intensity peak at 1645 cm^{-1}. A band centered at 1420 cm^{-1} has a shoulder near 1440 cm^{-1}. Two distinct peaks are at 1360 cm^{-1} and 1224 cm^{-1}, with weaker bands at 1092 cm^{-1} and 907 cm^{-1}. According to reference spectra, carotenoids and chlorophylls overlap in their IR spectra, making them difficult to distinguish. However, UV–Vis spectrophotometry provides more specific information on chlorophyll types (a/b) and relative carotenoid content.

UV- Vis Spectrophotometric Analyses

Results of the UV–Vis spectrophotometric analysis are shown in **Figure 8 and Table 2.**

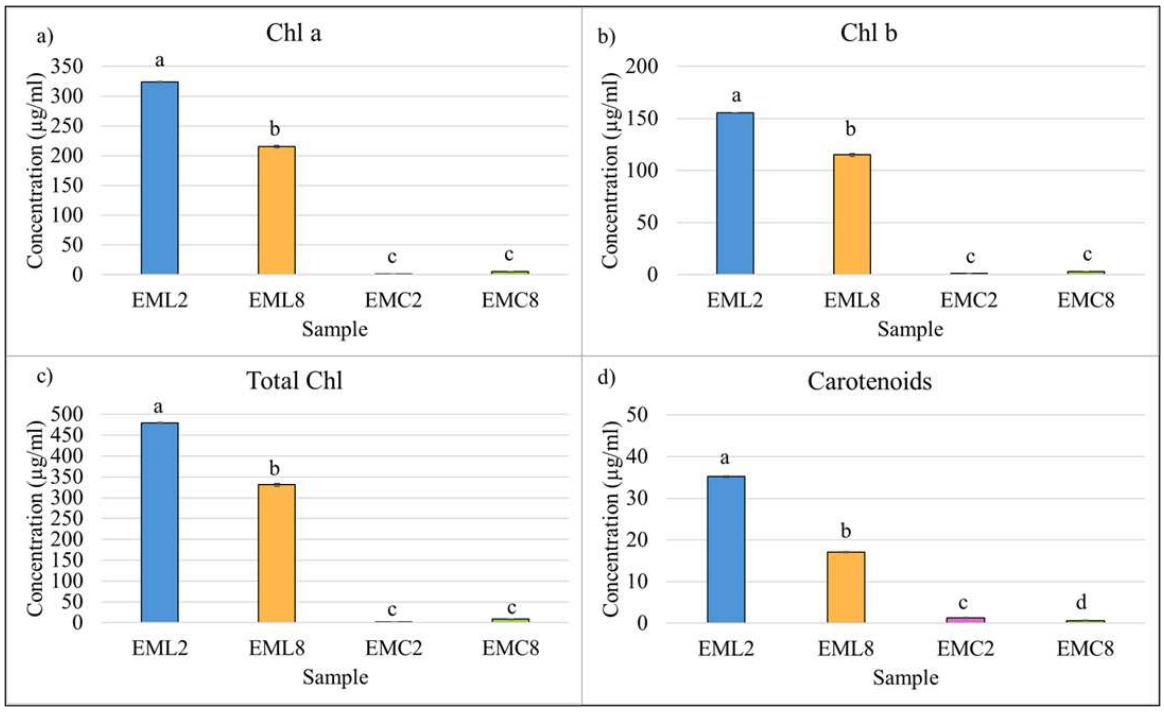


Figure 8. UV–Vis spectrophotometric analysis of chlorophyll and carotenoid contents in EML2 (blue), EML8 (orange), EMC2 (magenta), and EMC8 (green) samples. (a) Chlorophyll a (Chl a), (b) chlorophyll b (Chl b), (c) total chlorophyll (Chl a + b), and (d) carotenoids. Data are shown as concentrations (µg/mL) and are presented as means ± SD (n = 3). Different letters above bars denote statistically significant differences among groups according to Tukey's post hoc test following two-way ANOVA ($p < 0.05$).

Table 2. Shows UV–Vis quantification of chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophyll, and carotenoid concentrations in samples from EML2, EMC2, EML8, and EMC8 (μg/mL).

	EML2	**EML8**	**EMC2**	**EMC8**
Chl a	324.1 ± 1.4^{a}	215.8 ± 2.1^{b}	1.3 ± 0.0^{c}	5.4 ± 0.2^{d}
Chl b	155.4 ± 0.3^{a}	115.1 ± 1.3^{b}	1.2 ± 0.0^{c}	3.1 ± 0.1^{d}
Total Chl	479.4 ± 1.2^{a}	330.9 ± 3.3^{b}	2.5 ± 0.0^{c}	8.5 ± 0.3^{d}
Carotenoids	35.2 ± 0.2^{a}	17.1 ± 0.1^{b}	1.3 ± 0.1^{c}	0.7 ± 0.0^{c}

Chlorophyll a (Chl a) concentrations varied significantly among samples (two-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). Leaf samples formed two distinct statistical groups, with the highest Chl a content observed in EML2 (324.1 μg/mL; group A), followed by EML8 (215.8 μg/mL; group B), while both callus samples (EMC2: 1.3 μg/mL; EMC8: 5.4 μg/mL) clustered into a single statistically similar group (group C).

A similar pattern was observed for chlorophyll b (Chl b), with significantly higher levels in leaves than in calli. EML2 (155.4 μg/mL; group A) and EML8 (115.1 μg/mL; group B) were statistically different, while EMC2 (1.2 μg/mL) and EMC8 (3.1 μg/mL) were not significantly different from each other (group C).

Total chlorophyll (Chl a + Chl b) content followed the same statistical pattern, ranging from 479 μg/mL in EML2 (group A) to 2.5 μg/mL in EMC2 (group C), with EML8 forming an intermediate and significantly different group (group B).

In contrast, carotenoid levels varied significantly among the four samples, creating four distinct statistical groups. The highest carotenoid content was found in EML2 (35.2 μg/mL), followed by EML8 (17.1 μg/mL), EMC2 (1.3 μg/mL), and EMC8 (0.7 μg/mL), with no pairs being statistically similar ($p < 0.05$).

Overall, leaf samples (EML2 and EML8) contained significantly higher pigment levels than their respective calli. Chlorophyll a was the main pigment, followed by chlorophyll b (about 50% of Chl a in leaves) and carotenoids, resulting in an approximate ratio of 2:1:0.1 for Chl a: Chl b: carotenoids. Notably, EMC2 consistently showed low pigment levels, whereas EMC8 accumulated much more total chlorophyll, mainly Chl a, but had fewer carotenoids than EMC2. This suggests genotype-dependent differences in the regulation of pigment biosynthesis.

Discussion

Root and tuber crops are vital to the global food supply. The development of sweet potato storage roots is a complicated process involving interactions among multiple signaling pathways and various hormones [45]. The intracellular distribution of auxins and cytokinins across different cellular organelles has recently been detailed by Skalicky et al. [46] using fluorescence-activated mapping. Like in vivo plants, plant growth hormones are essential for cell division, organ differentiation, and overall plant growth in tissue cultures [37, 47]. However, the genetic background of hexaploid crops like sweetpotato is a crucial factor, as emphasized in numerous studies [10, 11, 12, 14].

The goal of this study was to evaluate the regenerative potential of protoplast cultures and their ability to trigger somatic embryogenesis as a regeneration method in the tested genotypes.

Unfortunately, this crop poses a challenge, and reproducible protocols for routine isolation and, specifically, for regenerating various genotypes are currently unavailable [48].
Results from our study also confirm that regeneration from a single-cell origin, such as protoplast cultures or meristematic tissue, is a complex process. Final success depends on carefully adjusting the composition of the culture media at various stages of in vitro plant development, and this is closely linked to the genotypes used in the experiment, as noted in the literature for different agricultural plant species, for example, by Foulger and Jones (1986), Golz et al. (1988), Barr et al. (1996), Reed and Bargmann (2021), and Jeong et al. (2024), among others.

As shown here, the initial cell division in protoplast cultures requires auxins and cytokinins at relatively low concentrations to trigger cell wall formation and the first divisions [38, 47]. Our results with protoplast isolation also suggest that, in the callus induction medium, auxins and cytokinins are essential (Figure 2g, h), as indicated by the first observed cell division. We will continue to optimize the entire process for specific genotypes, based on our callus induction and shoot regeneration results, and will incorporate new findings into this complex process. [49,50].

Results from the callus induction of 15 sweetpotato genotypes showed that both tested auxins (2,4-D and 2,4,5-T) had a very strong effect on both plant meristems, including the apical meristem and axillary buds, as shown in Table 3. Results from shoot induction are more variable, as shown in Figure 4. For shoot elongation, only the MS medium combined with kinetin and GA3 had a positive effect on shoot elongation in genotype LI1. In the remaining genotypes, even when several shoots were induced, they did not elongate or develop into normal *in vitro*-regenerated plants (Figure 3i,j).

Genotype EM 8 is also notable because the apical meristem and axillary buds readily achieve 100% callus induction. However, shoot induction was very limited, and no regular shoots developed, unlike the commercial variety Orleans, which exhibited higher shoot induction rates. Nonetheless, the induced shoots did not regenerate into normal in vitro plants. In both genotypes, we observed a strong presence of latex in the leaves and stem segments of donor plants (data not shown), which is likely to interfere with the culture medium, especially during the regeneration phase.

Callus induction of immature embryos from the genotypes EM2 and LI1, initially induced on MS medium supplemented with 2,4,5-T, resulted in organogenic callus. After a very long period (8 months), only genotype EM3 started to induce shoots on MS medium with kinetin. Later, these calli were transferred to MS medium supplemented with kinetin and GA_3, as shown in Figure 5h.
Histological results showed that the composition of the culture medium directly impacts tissue regeneration, as demonstrated by Adedeji et al. [51]. Our findings indicated that adding 2,4,5-T increased meristematic tissue formation in callus from two different lines, LI2 and HI2. The positive effect of auxins on callus development in other species was also noted (Zaranek et al. 2023)[52]. However, applying 2,4-D promoted morphogenesis and embryogenesis in calluses from the LI1, LI2, and EM3 lines, while no morphogenetic potential was observed in the HI1 and EM2 genotypes. This underscores the strong influence of genotypes. Additionally, histological sections of callus from the LI2 line revealed a high presence of meristematic and embryogenic cells on media supplemented with both 2,4-D and 2,4,5-T.
These findings suggest that genotypes have a greater impact on meristematic tissue formation than the growth regulators used. The dependence of callus regeneration ability on genotype was also found in species such as *Fagopyrum tataricum*, *Brassica oleracea*, *Daucus carota*, *Beta vulgaris* [53-56], as

well as *Linum usitatissimum* [57], *Stipagrostis pennata* [58], *Manihot esculenta* [59], *Oryza sativa* [60], *Triticum aestivum* [41], and several conifer species like *Larix* × *eurolepis* [61] or *Pinus roxburburii* [62].
The yellow-orange callus formation and its long-term proliferation without any signs of shoot induction or regeneration caught our attention. Callus from genotype EM2 and EM8 was analyzed for plant pigments (chlorophylls and carotenoids), since different pigments are observed in various sweetpotato leaves and stalks, even in callus derived from protoplast cultures [63-65].
Chlorophyll content is particularly relevant in this context, as photosynthetic activity drives glucose biosynthesis, which serves as the primary energy source and structural building block for plant growth and organ differentiation, including shoot regeneration. Carotenoids, which serve as accessory pigments in light harvesting and photoprotection, are also known as precursors in the ABA biosynthetic pathway in plants [66].
Because both plant pigments are found in the callus and leaf samples (Figure 8), their FTIR profiles are nearly identical, showing similar peak positions and intensities. The main difference appears at 963 and 948 cm^{-1} for β-carotenoids, linked to C=C stretching bands (950-961 cm^{-1}) typical of pure β-carotene [67]. However, these peaks are less distinct in mixed plant extracts due to chlorophyll dominance and matrix effects **[68].** Lutein and chlorophyll b were also used as reference compounds in the infrared analysis. Still, since lutein is a carotenoid and plant extract IR spectra have extensive band overlap, it isn't possible to reliably distinguish chlorophylls from lutein or other carotenoids using IR alone. Usually, separating chlorophyll a, chlorophyll b, and individual carotenoids requires chromatographic methods like HPLC with a diode-array detector [66]. Under our infrared measurement conditions, no clear spectral differences were seen between chlorophylls, lutein, and β-carotene within the IR region. Therefore, we used UV–Vis spectrophotometric analysis.
UV–Vis spectrophotometric analysis provided more accurate information about the types of chlorophyll (a/b) and the relative carotenoid content. EMC lines showed low concentrations of chlorophylls and carotenoids. However, EMC2 and EMC8 require further study to enable targeted production through metabolic engineering. Several optimization strategies aim to target calli with higher levels of carotenoids and/or chlorophylls. To increase β-carotene accumulation, reducing IbCHY-β expression in plant cell cultures proves effective, as shown by Kim et al**. [69].** This approach works particularly well when combined with salinity stress, which induces significant β-carotene production as a protective response against reactive oxygen species (ROS) [69, **70**]. Chlorophyll levels can be optimized by adjusting light exposure [71]. These strategies could improve yields for industrial applications.

Materials and Methods

Plant Material

Fourteen genotypes used in our experiments were initially obtained from the Gene Bank at the International Potato Center (CIP) in Lima, Peru. Slips of the commercial cultivar Orleans were purchased from Olssons Frö AB, Sweden. For more details, **see Table 3.**

Table 3. List of sweetpotato genotypes studied.

Genotype Name	CIP code	SLU code	Origin	Status	Flesh color
Benjamín	CIP105085.2	EM1	Peru	Variety	Orange
Arne	CIP105086.1	EM2	Peru	Variety	Orange
IDIAPC9017	CIP106090.1	EM3	Peru	Variety	Orange
IDIAPC0317	CIP106603.1	EM4	Peru	Variety	Orange
NN	CIP106906.1	EM5	Peru	Advanced clone	Orange
Abigail	CIP194540.5	EM6	Peru	Variety	Orange
NN	CIP199026.1	EM7	Peru	Advanced clone	Cream
Dagga	CIP199062.1	EM8	Peru	Variety	Yellow
Beauregard	CIP440132	HI1	USA/Peru	Variety	Orange
NN	CIP440185	HI2	USA/Peru	Advanced clone	Orange
NN	CIP194513.23	HI3	Peru	Advanced clone	Orange
NN	CIP194579.10	HI4	Peru	Advanced clone	Orange
NN	CIP105083.1	LI1	Peru	Advanced clone	Orange
NN	CIP190089.6	LI2	Peru	Advanced clone	Orange
Orleans	Orleans	Orleans	USA/Sweden	Variety	Orange

This material was surface-sterilized with 70% ethanol for 30 seconds, followed by a 20% Klorine© solution with 2 drops of Tween-20 for 20 minutes, then rinsed three times with sterile MQ water. *In vitro* cultures from all genotypes were established on hormone-free Murashige and Skoog MS medium **[42],** supplemented with 3% w/v sucrose and 0.3% w/v phytagel, at pH 5.6. All these *in vitro* cultures were regularly subcultured every 3- 4 weeks onto the same fresh MS hormone–free medium and maintained in long-term cultures in a Percival growth chamber (CLF Plant Climatic, Wertingen, Germany) at 23°C under a 16/8 photoperiod with a light intensity of 60 µmol $.m^{-2}$ s^{-1} and 60% relative humidity. All tissue culture media components, including growth regulators, were purchased from Duchefa (The Netherlands).

Protoplast Isolation and Cultivation

For protoplast isolation, leaves from three-week-old in vitro sweetpotato plants were collected following the protocol described by Dedicova et al. [**41**], with several modifications. The protoplast cultures were grown in the dark at 23°C with 60% relative humidity in a liquid medium containing ½ MS macronutrients and microelements, MS vitamins, 0.05 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid), 0,5 mg/L kinetin, 500 mg/L casein hydrolysate, 6 mM $CaCl_2.2H_2O$, 10 mM L-arginine, 1% sucrose, and 0.6 M D-mannitol. The pH was adjusted to 5.6, and the medium was filter-sterilized using a 0.2 µm syringe filter. The protoplast cultures were incubated at 23°C in the dark at 60% humidity in a Percival growth chamber (CLF Plant Climatic, Wertingen, Germany). For microscopic observation of the protoplast cultures, an inverted microscope (Leitz Diavert, Leica Microsystems, Stockholm, Sweden) with a camera system was used.

Callus Formation and Plant Regeneration from Stem Meristems

Apical meristems and axillary buds were isolated from three- to four-week-old in vitro sweetpotato plants (all 15 genotypes) grown on MS hormone-free medium supplemented with 30 g L^{-1} sucrose and 3 g L^{-1} phytagel, pH 5.6, using a Leica EZ4D stereo microscope (Micromedic AB, Stockholm, Sweden) in a laminar flow hood. These plants were maintained in a Percival growth chamber (CLF Plant Climatic, Wertingen, Germany) at 23°C under a 16/8 photoperiod with a light intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and 60% relative humidity. Explants were initially placed in MS medium (containing macronutrients, microelements, and vitamins) supplemented with 2 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) or 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), 30 mg L^{-1} sucrose, and 3 mg L^{-1} phytagel. This callus induction medium also contained 500 mg/L casein hydrolysate, 100 mg/L ascorbic acid, 1.25 mg/L $CuSO_4 \cdot 5H_2O$, 5 mg/L ACC (1-aminocyclopropane-1-carboxylic acid, Sigma-Aldrich, Sweden), 500 mg/L L-glutamine, and 500 mg/L L-proline. Before autoclaving, the medium pH was adjusted to 5.6. Components such as 2,4,5-T, ACC, L-proline, and L-glutamine were added to the final medium as filter-sterilized solutions. Cultures were incubated at 23°C in the dark with 60% humidity in a Percival growth chamber (CLF Plant Climatic, Wertingen, Germany).

After three weeks, the induced calli were transferred to fresh MS medium identical to the previous baseline medium, but supplemented with either 2 mg L^{-1} zeatin riboside or 2 mg L^{-1} kinetin. The cultures were then moved to a 16/8-hour photoperiod with a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$. All cultures were transferred to the same medium after 3- 4 weeks of growth. Well-developed cultures with induced shoots were transferred to shoot elongation MS medium supplemented with 5 mg L^{-1} kinetin, 1 mg L^{-1} GA_3, 500 mg L^{-1} L-proline, and 500 mg L^{-1} glutamine, along with 3% (w/v) sucrose and 0.3% phytagel, at pH 5.6. These were placed under a 16/8-hour photoperiod with a light intensity of 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Finally, elongated shoots were transferred to hormone-free MS medium supplemented with 3% (w/v) sucrose and 0.3% phytagel at pH 5.6. For all in vitro culture transfers, see Figure 3.

Embryogenic Callus Induction from Immature Embryos

Plant growth conditions were maintained under a 12-h photoperiod, with temperatures of 30 °C during the light period and 23 °C during the dark period. Relative humidity was maintained between 75–80%, and light intensity ranged from 250 to 300 $\mu mol. \, m^{-2} \, .s^{-1}$. A commercial substrate (SW Horto AB) was used, and fertilization was supplemented with Solinure GT (NPK 12–5–35).

Immature embryos from two genotypes (EM3 and LI1) were collected two months after flowering began. Capsules containing immature seeds and embryos were gathered into a 50 mL Falcon tube. This material was surface-sterilized with 70% (w/v) ethanol for 30 seconds, followed by a 20% (w/v) Klorine© solution (containing 2.7% (w/v) active chlorine, Colgate-Palmolive AB, Sweden) with two drops of Tween-20 for 20 minutes, then rinsed three times with sterile Milli-Q water. Immature embryos were dissected under a Leica EZ4D stereo microscope (Micromedic AB, Stockholm, Sweden) on a laminar-flow bench and placed in callus-induction medium, scutellum-side up. They were initially cultured in MS medium (containing macronutrients, microelements, and vitamins) supplemented with 2 mg L^{-1} of 2,4-D (2,4-dichlorophenoxyacetic acid) or 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), 3% (w/v) sucrose, and 0.3% (w/v) phytagel. This callus induction medium also included 500 mg L^{-1} casein hydrolysate, 100 mg L^{-1} ascorbic acid, 1.25 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 5 mg L^{-1} ACC (1-aminocyclopropane-1-carboxylic acid, Sigma-Aldrich, Sweden), 500

mg L^{-1} L-glutamine, and 500 mg L^{-1} L-proline. Before autoclaving, the pH was adjusted to 5.6. Components such as 2,4,5-T, ACC, L-proline, and L-glutamine were added to the final medium as filter-sterilized solutions. Cultures were incubated at 23°C in the dark with 60% humidity in a Percival growth chamber (CLF Plant Climatic, Wertingen, Germany).

Histological Studies

Samples of three to five callus clumps collected from the callus induction medium MS supplemented with 2,4-D or 2,4,5-T, three weeks after subculture, were fixed in freshly prepared 100 mM phosphate buffer (pH 7.2) containing 2% (w/v) formaldehyde (POCH) and 3% (w/v) glutaraldehyde (POCH) for at least 48 hours at room temperature, with vacuum infiltration during the first 10 minutes. After fixation, the callus samples were dehydrated through an ethanol series (10%, 30%, 50%, 70%, and 90% for 15 minutes each) and left overnight in absolute ethanol. The dehydrated material was embedded in Technovit® 7100 resin (Kulzer, Germany) following the manufacturer's protocol. Once polymerized, the samples were sectioned into 4 μm slices using a Leica RM2145 rotary microtome (Leica Microsystems GmbH, Germany) with a Leica TC-65 carbide blade. The sections were then stained with 1% (w/v) toluidine blue O (Sigma-Merck, Germany), permanently mounted in Entellan® (Sigma-Merck, Germany), and examined under an Axio Imager.M2 microscope (Carl Zeiss, Germany).

Plant Pigment Content Measurement

In vitro callus cultures were collected under a laminar flow hood. The selected calli were roughly chopped with a cutter. The samples included early-maturing calli (EMC) genotypes EM2 and EM8, along with their leaves (EML) as controls, for a total of 4 samples (Table 4).

Table 4. Sample labeling for FTIR and UV-Spectrophotometry.

Leaves (control)	Callus (experimental conditions)
EML2	EMC2
EML8	EMC8

The calli were then immediately frozen and freeze-dried overnight before being ground with a mortar. Extraction was performed on 50 mg of ground sample mixed with 2 mL of 80% acetone, and the mixture was centrifuged at 12,000 g for 10 minutes at 4°C **[72,73].** The supernatant was directly analyzed using FTIR and UV-Vis spectrophotometry.

Fourier Transform Infrared (FTIR) Analysis

Infrared analysis was performed on extracted carotenoids and chlorophylls from calli samples (supernatant from section 2.6), and the results were compared with those of the corresponding leaves used as controls.
The targeted samples included early-maturing calli (EMC) genotypes EM2 and EM8, along with their leaves (EML) as controls, for a total of 4 samples (Table 2).

Infrared spectroscopy identifies the molecular composition of samples by analyzing the types of bonds between molecules. A small amount of the liquid supernatant was placed on the ATR crystal, and spectra were recorded using an Agilent Cary 630 spectrometer (Table 2). Spectra were collected

after 64 scans across the spectral range of 800–4000 cm^{-1} for each sample. The β-carotene (Sigma-Aldrich, Sweden) and chlorophyll a (Sigma-Aldrich, Sweden) were analyzed and used as standards to identify carotenoids and chlorophylls.

Spectrophotometer UV-Vis

To determine the concentrations of chlorophylls and carotenoids, 300 μL of the previously obtained supernatant was transferred to a 96-well plate. The absorbance was measured at 470, 646, and 663 nm (A470, A646, and A663, respectively). A646 and A663 correspond to the maximum absorbance (Amax) in 80% acetone according to Lichtenthaler [74], using a Thermo Scientific Multiskan GO Spectrophotometer and the following formula based on the Beer-Lambert law [75]:

$$Chl\ a = (12.25 * A_{663}) - (2.79 * A_{646})$$

$$Chl\ b = (21.5 * A_{646}) - (5.10 * A_{663})$$

$$Carotenoids = \frac{(1000 * A_{470} - (1.82 * Chl\ a) - (85.02 * Chl\ b)}{198}$$

Chl a and Chl b represent chlorophyll a and chlorophyll b, respectively. The values 12.25 and 21.5 indicate the specific absorbance of Chl a at 663 nm and Chl b at 646 nm, respectively. The values 2.79 and 5.10 are used to correct for Chl b contribution at 663 nm and Chl a at 646 nm. These are derived from absorbance measurements in 80% acetone using the Beer-Lambert law. The results are expressed in μg/ml.

Statistical Analysis and Data Visualization

Callus formation, shoot induction, and shoot regeneration were analyzed using a generalized linear model (GLM) assuming a Poisson error distribution and a logarithmic link function, with the number of shoots formed as the response variable. To account for differences in the number of calli evaluated among experimental units, the log-transformed number of cultivated calli was included as an offset term, enabling the modeling of shoot formation rates per callus.

The model included sweetpotato variety, explant type (apical and axillary), hormonal treatment, and the explant type × treatment interaction as fixed effects. The significance of model terms and interactions was evaluated using analysis of deviance and likelihood-ratio chi-square tests to compare nested models. This analysis helped determine the relative contributions of each factor and its interactions to shoot formation. The unbalanced experimental design, due to variation in the number of calli evaluated across treatments and varieties, was effectively addressed within the GLM framework.

When significant effects were observed, estimated marginal means were calculated on the response scale, and multiple comparisons among varieties within each combination of explant type and hormonal treatment were performed using Tukey's adjustment at α = 0.05. Results are shown as estimated shoot-forming rates per callus, along with 95% confidence intervals.

To support the interpretation of multidimensional response patterns, a three-dimensional scatter plot was created using model-adjusted estimates from the Poisson GLM. In this visualization, variety and hormonal treatment were assigned to the horizontal axes, shoot formation rate per callus to the vertical axis, and explant type was differentiated by color. This graphical representation was used for exploratory and descriptive purposes. All statistical analyses and visualizations were conducted in R (Table 5).

Table 5. Deviance analysis (χ^2 tests) for shoot formation per callus in sweetpotato, assessing the effects of variety, explant type, hormonal treatment, and their interactions.

Effect	Df	Deviance	Pr(>χ^2)
Variety	12	73.74	<0.001***
Explant type	1	0.25	0.616
Treatment	3	4.51	0.211
Explant × Treatment	3	11.12	0.011*

Conclusions

This study, consistent with previous research, confirms that genotypes significantly affect sweetpotato shoot induction and in vitro plant regeneration. Both tested auxins, 2,4-D and 2,4,5-T, yielded similar results for callus formation and growth, with Orleans and HI2 the most effective genotypes for embryogenic callus induction. These two regeneration methods were validated by histological analysis. During shoot induction and proliferation, kinetin-containing media were more effective than zeatin-containing media in promoting the formation of original shoot meristems in the presence of 2,4,5-T. Some lines failed to induce shoots despite prolonged culture and transfers with these two cytokinins (Zeatin or Kinetin). Additionally, BAP had no positive effect on shoot induction in certain non-responsive genotypes (data not shown). Notably, immature embryos from two genotypes (EM3 and LI1) did not form embryogenic callus but instead regenerated via organogenesis, producing multiple shoots (EM3). Extensive research on sweetpotato protoplast isolation indicated that cell division occurred only in the EM5 genotype. Analysis of pigment composition in yellow-orange callus from EM2 and green callus from EM8 provided further insights. These findings will support future efforts to preselect genotypes for sweetpotato protoplast culture protocol development. All data obtained in this study will serve as a foundation for preselecting genotypes for future research on sweetpotato protoplast culture and, potentially, for establishing the editing technology, among other applications, where the top-performing genotype still needs to be identified within this breeding material.

Data Availability

All original results from this study are included in this article.

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Author Contributions

B.D. - Conceptualization, methodology, formal analysis, investigation, visualization, and original draft writing. F.D.T. - Data analysis and original draft writing (statistics). E.G. - Investigation, methodology (histology), and original draft writing. E.M. - Investigation, visualization (histology), and original draft writing. M.S.D. - Investigation, methodology (plant pigments), and original draft writing. N.L.E. and D.E. - Funding acquisition. All authors have read and approved the final manuscript.

Declarations

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Conflicts of Interest

The authors declare no conflicts of interest.