Chapter

In Vitro Applications in Cucumber (*Cucumis sativus* L.)

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Abstract

Cucumber (*Cucumis sativus* L.), a vital horticultural crop in the Cucurbitaceae family, has been widely studied for its physiological, developmental, and genetic traits. *In vitro* culture techniques have played a critical role in facilitating genetic improvement, trait selection, and propagation of elite lines. This review comprehensively discusses the major *in vitro* techniques applied in cucumber research, including callus induction, shoot organogenesis, somatic embryogenesis, haploid production, and protoplast culture. Among various explants used, cotyledons, hypocotyls, and shoot apices are the most responsive for regeneration. Optimization of culture media especially the use of cytokinins (BAP, kinetin) and auxins (NAA, IAA) has significantly enhanced regeneration rates. In recent years, genetic transformation using *Agrobacterium tumefaciens* has gained prominence, particularly when coupled with *in vitro* regeneration systems. Furthermore, CRISPR/Cas9 genome editing has revolutionized cucumber biotechnology by enabling precise modifications for disease resistance, abiotic stress tolerance, and metabolic enhancements. Applications targeting genes such as MLO, eIF4E, and CsSGR have demonstrated remarkable potential in generating resistant and stress-tolerant cultivars. Hairy root systems have emerged as rapid platforms for functional gene analysis, especially for root-related traits. This chapter highlights the advancements and challenges in utilizing tissue culture and genome-editing techniques for improving cucumbers. Continuous innovations in explant selection, transformation efficiency, and regeneration protocols are essential to fully harness the crop's genetic potential.

Keywords: explant, micropropagation, organogenesis, plant regeneration, somatic embryogenesis

1. Introduction

Cucumber (*Cucumis sativus* L.) (2n = 2x = 14), which is a member of the diverse Cucurbitaceae family, is a widely cultivated vegetable crop with significant economic value worldwide [1]. It yields nutritious fruits rich in proteins, minerals, soluble carbohydrates, and vitamins. The fruit's polysaccharides and flavonoids are essential

for antioxidant functions, cancer prevention, and managing liver inflammation [2]. A range of biotic and abiotic stresses often impact cucumber production. Significant challenges include insect pests and diseases, as well as nutrient deficiencies and adverse weather conditions, such as drought or extreme temperatures. These stresses can significantly reduce cucumber yields and even deteriorate fruit quality [3]. Cucumber cultivation suffers significant losses due to these challenges—some research suggests that combined biotic and abiotic stresses can lead to yield reductions of up to 80–97% in severe situations [3]. Traditional breeding approaches, such as crossbreeding (hybridization between distinct cucumber lines) and mutation breeding (inducing heritable changes through physical or chemical mutagens), have served as essential tools in cucumber (*Cucumis sativus* L.) improvement programs. Over recent decades, these conventional methods have significantly contributed to the development of new cultivars characterized by enhanced yield, fruit quality, and market adaptability. Traditional genetic breeding has laid the groundwork for many of the high-yielding, superior-quality cucumber varieties cultivated globally today [4]. However, despite these achievements, the advancement of traditional breeding remains inherently slow, often requiring multiple generations to stabilize desirable agronomic traits. A major limitation in cucumber breeding is its inherently narrow genetic base. Due to historical domestication bottlenecks, the species exhibits limited genetic diversity and a low level of natural variation within its germplasm [5]. This restricted genetic pool poses challenges in achieving sustained genetic gains and limits the breeder's ability to incorporate novel traits. Consequently, breeding programs may encounter a plateau in improvement unless innovative strategies—such as the integration of wild relatives, molecular tools, or tissue culture techniques—are employed to broaden genetic variation or overcome inherent limitations in the cucumber gene pool [6].

Plant tissue culture has emerged as a powerful adjunct to cucumber breeding, helping to address the above constraints in several ways. First, micropropagation techniques enable rapid clonal multiplication of desired cucumber genotypes, producing large numbers of identical plants in a short time frame and space, irrespective of season [7]. This is particularly valuable for propagating elite F_1 hybrids or genetically superior lines that cannot be easily maintained by seed due to segregation. Studies have shown that cucumber plants regenerated *in vitro* can retain genetic fidelity to the mother plant. For instance, regenerants derived via somatic embryogenesis have shown no significant molecular differences from the original F_1 hybrid, confirming the maintenance of genetic stability through the tissue culture process [8]. Such clonal propagation ensures that breeders can rapidly produce uniform planting material (disease-free and true-to-type), thereby overcoming the slow generation turnover of conventional breeding.

Tissue culture forms the basis for modern biotechnological techniques. An effective *in vitro* regeneration system is essential for genetic transformation in cucumber—introducing new genes, such as those for virus resistance or stress tolerance, through *Agrobacterium*-mediated transfer, depends on the ability to regenerate whole plants from transformed cells [9]. In practice, cucumber's genotype dependence in regeneration posed a challenge, but optimized tissue culture protocols (utilizing responsive cultivars like Poinsett 76 or 9930) have allowed researchers to produce transgenic cucumber plants routinely. This capability enables precise genetic enhancements that surpass the limitations of a limited gene pool, allowing the introduction of new traits or gene edits that crossing alone cannot achieve [10].

In vitro selection techniques, combined with tissue culture, can help develop stress-tolerant lines by exposing cell cultures or regenerating plantlets to selective agents such as high salinity or pathogens [11]. This process enables breeders to identify and rescue rare genetic variants with improved tolerance. Moreover, screening for desirable traits *in vitro* is much faster and requires less space than traditional field-based methods, accelerating the development of traits like disease or salt resistance [12]. Additionally, somaclonal variation observed during cucumber tissue culture, once seen as a mere drawback, is now intentionally utilized under controlled selection pressure to produce novel variation for crop improvement [10, 12, 13].

Haploid and doubled haploid (DH) plant production has emerged as a powerful tool in modern cucumber breeding programs, particularly in overcoming the limitations of traditional selection methods. Through tissue culture-based approaches such as anther culture (androgenesis) and irradiated pollen-induced gynogenesis, breeders can rapidly obtain completely homozygous lines within a single generation, significantly accelerating breeding cycles [14, 15]. Additionally, protoplast culture in *Cucumis sativus* represents a versatile *in vitro* technique that enables cell wall removal and subsequent regeneration into whole plants. Protoplast systems have been used for haploid and mixoploid isolation, as well as PEG-mediated somatic hybridization involving cucumber and related *Cucumis* species, enabling novel genetic combinations not achievable through sexual crossing [16].

Due to the limitations of traditional breeding and recent advances in biotechnology, *in vitro* tissue culture techniques have become essential in modern cucumber improvement. Main regeneration methods—somatic embryogenesis, organogenesis, and haploid plant production—allow for genetic fidelity, efficient shoot regeneration, and quick achievement of homozygosity, respectively [8, 9, 14]. Micropropagation supports large-scale multiplication of elite, disease-free lines [7, 17], while protoplast culture shows promise for genetic manipulation and somatic hybridization [18]. This chapter aims to offer a comprehensive overview of the key *in vitro* tissue culture techniques used to improve cucumber.

2. *In vitro* systems

2.1 Somatic embryogenesis

Somatic embryogenesis (SE) is a pivotal regeneration pathway in cucumber, enabling efficient clonal propagation, facilitating genetic transformation, and contributing to the development of stress-tolerant cultivars (**Figure 1**). The success of SE is strongly influenced by factors such as genotype, explant type, and *in vitro* culture conditions. Genotypic effects play a crucial role as limiting factors. Hamza, Diab [19] found that the Egyptian cultivar Beit Alpha could produce up to 60% of embryogenic callus from cotyledon-leaf explants, whereas the cultivar Prince only reached about 40%, emphasizing the importance of genotype in regeneration success. Similar differences in SE potential among various cultivars have been consistently documented in the existing literature [20, 21]. Cotyledons are the most frequently used and responsive explants for SE induction, providing high embryogenic potential when cultured on media enriched with Ref. [8, 22]. Hypocotyls and leaf segments also demonstrate potential, though they typically need more precise hormone balances to induce embryogenic callus [23, 24]. Additionally, shoot tips, mature seeds,

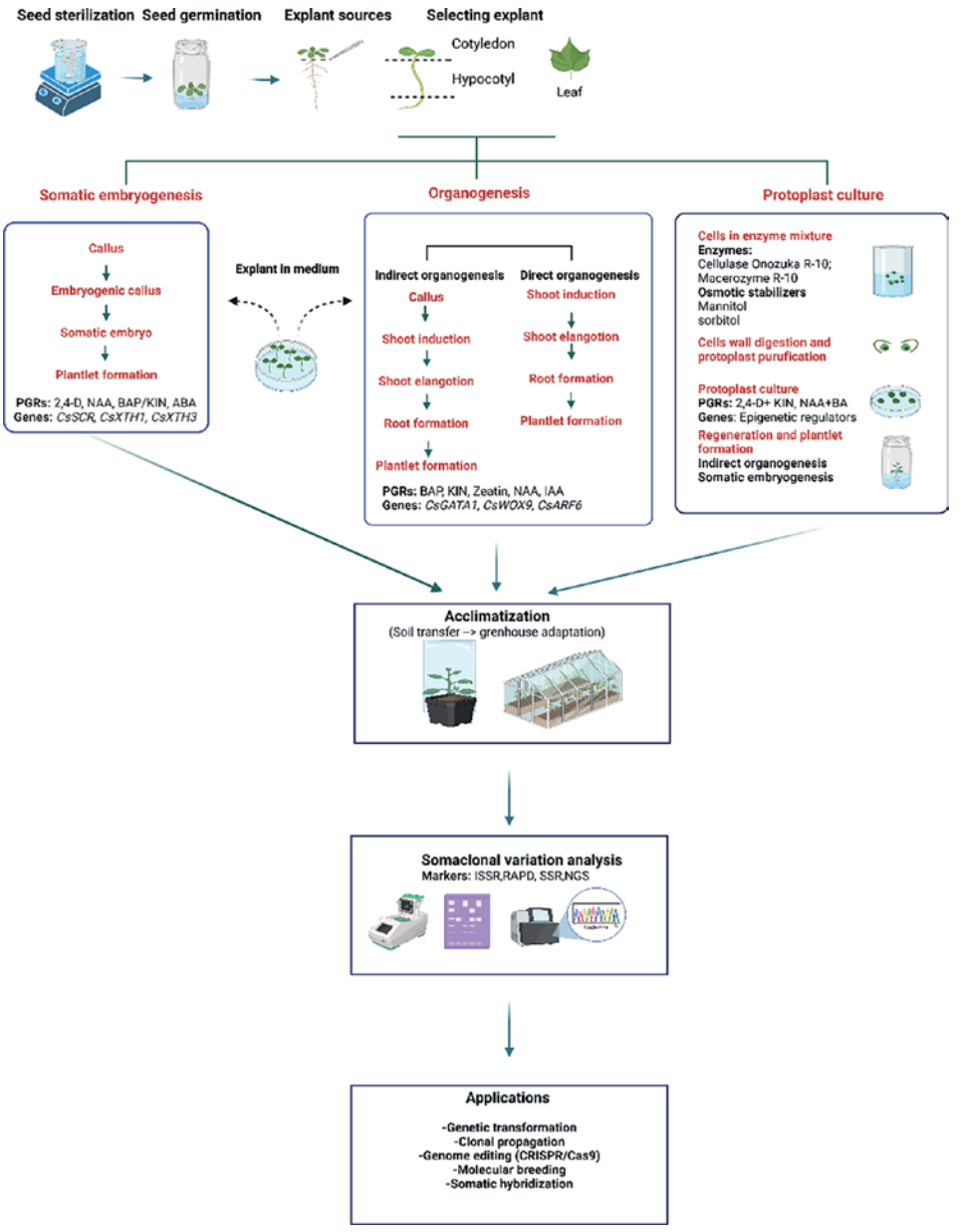

Figure 1.
*In vitro regeneration pathways in cucumber (*Cucumis sativus *L.) through somatic embryogenesis, organogenesis, and protoplast culture, followed by acclimatization, somaclonal variation analysis, and applications. Created with BioRender.com.*

and cotyledon-node regions have been studied, showing varied responses influenced mainly by the cucumber cultivar and the explant's developmental stage [25, 26].

Plant growth regulators (PGRs) are crucial for controlling somatic embryo initiation, development, and maturation. 2,4-Dichlorophenoxyacetic acid (2,4-D) remains the most common auxin used for SE induction in cucumber. Studies show that 2–5 mg/L of 2,4-D significantly encourages callus formation and early embryo

development, especially in cotyledon and leaf explants [24, 27]. Naphthaleneacetic acid (NAA) is also used, often in combination with cytokinins such as benzylaminopurine (BAP) or kinetin (KIN), to enhance callus proliferation and facilitate the transition to somatic embryos [20, 26]. The latter stages of embryo maturation and shoot conversion typically require a hormonal shift toward cytokinin dominance. The use of abscisic acid (ABA) has also been shown to promote maturation and desiccation-like responses, enhancing the quality of somatic embryos [28]. Additionally, carbohydrate sources with high sucrose levels (up to 12%) not only supply energy but also create osmotic stress that mimics zygotic conditions, further stimulating embryogenesis [22, 29]. Additionally, bioreactors like RITA® have facilitated scaled-up SE systems in elite hybrids [30].

At the molecular level, several genes (e.g., *CsSCR*, *CsXTH1*, *CsXTH3*) and proteins have been identified as being upregulated during somatic embryo induction [21, 31]. Additionally, studies using inter-simple sequence repeats (ISSR) markers, RT-qPCR, and hormone profiling have clarified the transcriptional and hormonal pathways active during dedifferentiation and redifferentiation [28, 31, 32]. CsSCR (SCARECROW) is a GRAS-family transcription factor strongly upregulated in globular- and heart-stage somatic embryos and in rooting tissues. Wisniewska and Grabowska [31] showed elevated CsSCR transcript levels, confirmed via in situ hybridization and expression assays, implicating its role in radial tissue organization during embryo formation. Malinowski and Filipecki [33] conducted a comprehensive study on two xyloglucan endotransglucosylase/hydrolase (XTH) genes, *Cs-XTH1* and *Cs-XTH3,* in *Cucumis sativus*. These enzymes remodel cell walls by cutting and rejoining xyloglucan chains, a critical process during embryo formation. Moreover, both genes showed elevated transcript levels in somatic embryos compared to pre-embryogenic tissue. Northern blot and *in situ* hybridization revealed Cs XTH1 localized in presumptive cotyledon primordia, while Cs XTH3 was also active during embryonic development.

2.2 Organogenesis

Organogenesis is a fundamental *in vitro* regeneration strategy in cucumber, employed widely for clonal propagation and genetic transformation (**Figure 1**). The efficiency of organogenesis is influenced by several interacting factors, including the type of explant, culture medium, PGRs, genotype, and environmental conditions [4]. Genotypic variation plays a critical role in the success of organogenesis in cucumber. Different cultivars exhibit significant differences in their regenerative responses under identical *in vitro* conditions. Some genotypes exhibit high shoot regeneration frequencies from specific explants, such as cotyledons or shoot tips, while others respond poorly, even with optimized media and PGR. This variation is likely due to underlying genetic control of hormonal sensitivity, developmental plasticity, and expression of key regulatory genes. Wang and Zhou [34] identified candidate genes (e.g., CsGATA1) and quantified trait loci (QTLs) associated with regeneration ability, underscoring the importance of genotype selection and molecular screening in developing efficient organogenic protocols for cucumber improvement. Nie and Shan [35] demonstrated that floral organogenesis is regulated via the CsDFB1–CsPHB module, which influences local auxin distribution. Slomnicka and Cieplak [21] identified QTLs such as sr6.1A and sr6.1B for shoot regeneration from leaf explants and highlighted CsWOX9 and CsARF6 as candidate genes for organogenic control.

Among explants, cotyledonary nodes and cotyledons are the most responsive. Selvaraj and Vasudevan [36] achieved up to 86% of shoot regeneration from cotyledon explants using MS medium with BAP, NAA, zeatin, and glutamine. Liu [37] demonstrated that cotyledon nodes yielded a higher adventitious bud induction rate compared to true leaves, radicles, and hypocotyls. Zheng [38] reported that changing the explant source from stem nodes to cotyledons improved the bud induction rate from 63.5% to 78.3%. Similarly, Venkatachalam and Jinu [7] demonstrated over 50 shoots per explant using 1.5 mg/L BAP + 4.5 mg/L silver nitrate in cotyledonary nodes, confirming their high morphogenic capacity. Hypocotyls and shoot tips are also used. Sangeetha and Venkatachalam [39] reported direct shoot formation and *in vitro* flowering from shoot tips on BAP-enriched medium. Although explants such as hypocotyls and leaves can also support regeneration, they generally exhibit lower efficiency than cotyledon-derived tissues [4]. Furthermore, the explant's physiological age is crucial in determining its regenerative ability. Young seedlings, typically 1 to 7 days old, are generally more suitable for organogenesis because their cells are less differentiated and respond more effectively to *in vitro* signals. Explants at these early stages can improve shoot induction rates and overall transformation success, owing to increased cellular plasticity and hormonal responsiveness [40].

PGRs play a crucial role in initiating and directing the organogenic process. Cytokinnins such as BAP, KIN, and zeatin stimulate shoot bud formation, while auxins like NAA and indole-3-acetic acid (IAA) help in callus induction and shoot elongation [41]. For hypocotyls, optimal shoot regeneration was achieved using 3.6 μM 2,4-D and 2.2 μM BAP [36]. ABA has been reported to prevent vitrification and improve organogenesis quality [4]. Moreover, silver nitrate enhances shoot quality by mitigating the effects of ethylene [42]. Venkatachalam and Jinu [7] showed that a medium containing 1.5 mg/L BAP and 4.5 mg/L silver nitrate significantly improved shoot proliferation from cotyledonary node explants, yielding over 50 shoots per explant and reducing hyperhydricity. In addition, environmental factors also play critical roles in plant regeneration. Temperature, gelling agent type, and photoperiod length have been shown to influence morphogenesis outcomes [43]. Light-induced transcriptional responses and NO-mediated cell cycle activation also contribute to shoot organogenesis in cucumber [44, 45].

2.3 Haploid culture

Haploid and DH production techniques have revolutionized plant breeding by enabling the rapid fixation of homozygosity within a single generation, significantly accelerating the development of pure lines. In cucumber, a cross-pollinated and highly heterozygous species, generating haploid and DH plants, offers substantial advantages for hybrid seed production, QTL mapping, mutation screening, and functional genomics. Although these methods are well established in cereals and solanaceous crops, their efficient implementation in cucurbits has been more challenging due to species-specific resistance, low regeneration rates, and strong genotype dependence [46, 47]. Nevertheless, two decades of intensive research have led to significant advances in haploid induction, utilizing anther culture, ovary culture, and parthenogenesis, supported by improvements in explant selection, media formulation, pretreatment protocols, and molecular techniques validation (**Figure 2**).

Among these approaches, anther culture remains one of the most studied methods for inducing haploid formation via androgenesis. Microspores at the uninucleate to early binucleate stages, typically found in 2–4 mm long floral buds, are considered

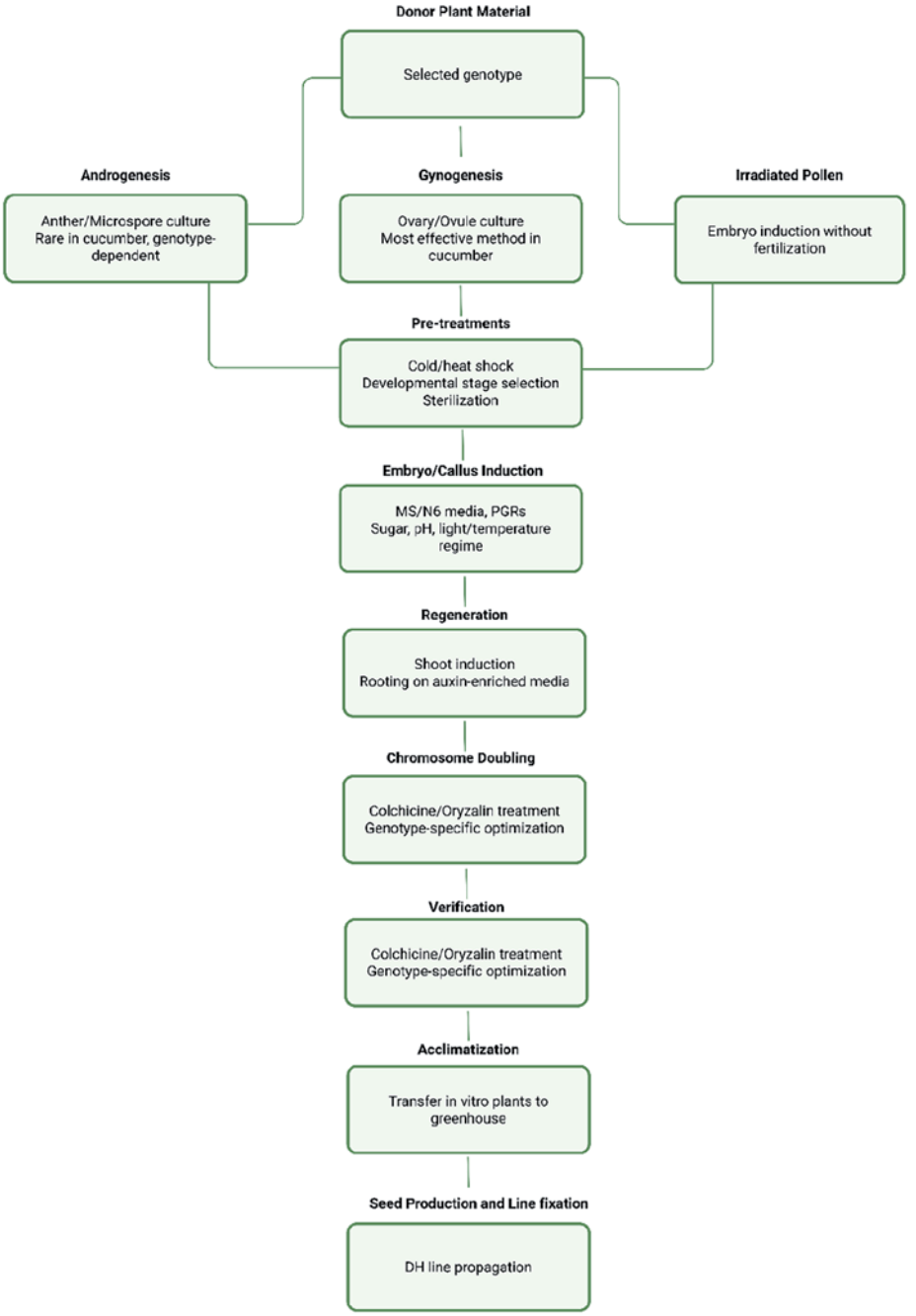

Figure 2.
*Doubled haploid (DH) production in cucumber (*Cucumis sativus *L.). Created with BioRender.com.*

most responsive to *in vitro* embryogenic induction [14, 48]. However, cucumber is a recalcitrant species for androgenesis due to the frequent formation of non-embryogenic callus and albino plantlets [49, 50]. These limitations have stimulated the exploration of alternative explants and pathways. Gynogenesis, through the

culture of unpollinated ovaries or ovules, has emerged as a viable and sometimes superior alternative, especially for genotypes with poor androgenic response. Ovary explants collected 1 to 3 days after anthesis have shown high embryogenic potential under appropriate culture conditions [51, 52]. Domblides and Shmykova [53] further demonstrated the success of unpollinated ovule culture in various cucurbit species, reinforcing the relevance of gynogenesis for haploid production. Furthermore, *in vitro* ovary culture can circumvent the developmental limitations of male gametophyte-derived explants, especially in genotypes that are poor responders to another culture [51, 54]. In addition to androgenesis and gynogenesis, parthenogenesis, embryo development from unfertilized egg cells, represents another viable strategy for haploid production. Parthenogenetic haploid embryos can be induced either *in vivo*, using irradiated pollen or distant hybridization (e.g., melon × cucumber), or *in vitro* through hormone treatments. Parkash and Bhatia [55] reported an efficient protocol for *in vitro* induction of parthenogenetic embryos using hormone-enriched MS media. Similarly, Salehian and Shahnazi [56] achieved DH plant production through parthenogenesis combined with precise embryo rescue techniques. These approaches are particularly advantageous in breeding programs aiming to develop DH lines for hybrid seed production. In particular, the application of cycocel (CCC) in combination with polyamines in the culture medium has been effective in stimulating parthenogenesis and improving the regeneration of haploid plantlets [57].

The choice of culture medium and hormonal balance plays a central role in determining the success of haploid induction. Murashige and Skoog (MS) medium remains the most commonly used basal medium, although modifications such as reducing nitrate concentration or adding vitamins (e.g., thiamine, myo-inositol) have been tested to optimize callogenesis and embryogenesis [51]. PGRs are critical for directing the developmental pathway of explants. Cytokinins such as BAP, KIN, and especially thidiazuron (TDZ) have been widely used to enhance shoot regeneration and embryogenic callus formation. The use of MS medium supplemented with silver nitrate, TDZ, and KIN has been shown to promote gynogenic embryo induction [58]. On the auxin side, NAA and 2,4-D are often employed during the callus induction phase to support cell dedifferentiation and proliferation [51]. Moreover, the incorporation of organic additives such as casein hydrolysate, coconut water, or activated charcoal has shown potential in improving the quality and frequency of embryogenic cultures [57]. These components help stabilize the pH, absorb phenolic compounds, and provide additional nutrients or growth-promoting substances.

In vitro embryogenesis is often facilitated by the application of stress treatments that redirect gametophytic development toward sporophytic pathways. Cold pretreatment, involving exposure of flower buds or ovary explants to low temperatures (typically 4°C) for 2–7 days, has consistently been shown to enhance embryogenic potential in cucumber [59]. The mechanism is believed to involve stress-induced expression of embryogenesis-related genes and hormonal reprogramming. Additionally, osmotic stress through the use of high sucrose or mannitol concentrations, or heat shock treatments, can improve embryogenic outcomes by inducing physiological changes in microspores or ovular tissues. In androgenesis protocols, a short heat shock at 32–35°C is sometimes applied prior to culture initiation [60].

For the practical application of haploid plants in breeding, chromosome doubling is required to restore fertility. This is typically achieved through the application of antimitotic agents such as colchicine, oryzalin, or trifluralin, which disrupt spindle formation during mitosis. Ebrahimzadeh and Shariatpanahi [57] demonstrated successful chromosome doubling in cucumber haploids by soaking regenerated

shoots in colchicine or trifluralin solutions, achieving a high percentage of fertile, morphologically normal DH plants. Shoot tip immersion and *in vitro* treatments are commonly used to avoid toxicity and ensure uniform exposure. The timing and duration of exposure must be carefully optimized to balance efficacy with plant survival. Also, verification of haploid and DH status is a crucial step in haploid breeding. Flow cytometry is a rapid and reliable method for ploidy analysis, allowing high-throughput screening of regenerated lines. Molecular markers such as simple sequence repeats (SSRs), random amplified polymorphic DNA (RAPD), and ISSRs are also employed to assess homozygosity and confirm the genetic uniformity of DH lines [47, 61].

2.4 Protoplast culture

Protoplast culture in cucumber has emerged as a powerful biotechnological tool for genetic transformation, somatic hybridization, and *in vitro* regeneration, despite being limited in routine applications due to technical complexities and genotype-dependent variability (**Figure 1**) [4, 62]. Protoplasts, plant cells devoid of their cell walls, serve as an excellent model system for studying cellular totipotency, early morphogenesis, and cell wall regeneration under controlled *in vitro* conditions [18, 63]. Studies have demonstrated successful plant regeneration via direct somatic embryogenesis and organogenesis from mesophyll and cotyledon-derived protoplasts in cucumber. However, the frequency and stability of regeneration are often highly genotype-specific [64, 65].

Efficient isolation and culture of cucumber protoplasts have been reported using the enzymatic digestion of cotyledon or mesophyll tissues. Huang and Wang [18] developed a high-yield protoplast isolation method using a combination of 1.5% cellulase R10 and 0.4% macerozyme R10, supplemented with 0.6 M mannitol as an osmotic stabilizer in an enzyme solution buffered with MES hydrate (2-(N-morpholino hydrate) ethane sulfonic acid at pH 5.7. The digestion was conducted under dark conditions with gentle shaking at 25°C for 6 hours, resulting in highly viable protoplasts suitable for transient gene expression and downstream applications. Similarly, Punja and Tang [62] achieved successful isolation and regeneration of protoplasts derived from both mesophyll and cotyledon tissues using a two-enzyme mixture of cellulase and pectinase in a modified KM (kao and michayluk) medium with 0.4–0.5 M sorbitol. Their results demonstrated that shoot regeneration was possible through plating protoplasts onto solidified KM medium enriched with appropriate PGRs, such as 1 mg/L NAA and 0.5 mg/L BA. Furthermore, Burza and Malepszy [64] reported successful regeneration via direct somatic embryogenesis from cucumber protoplasts isolated using 1% cellulase Onozuka R-10 and 0.5% pectinase, suspended in an osmoticum containing 0.5 M glucose. They highlighted that embryogenic calli could be induced on N6-based medium supplemented with 2,4-D (2.0 mg/L) and KIN (0.5 mg/L), followed by shoot regeneration upon transfer to a cytokinin-enriched MS medium. These studies collectively emphasize the critical role of enzyme composition, osmotic balance, and hormone supplementation in establishing effective protoplast culture and regeneration systems in cucumber.

Protoplast regeneration is highly genotype-dependent, and the ability to form callus or regenerate shoots varies significantly among cucumber cultivars. Gajdová and Navrátilová [66] emphasized the importance of donor tissue type, culture medium composition, and genetic background in determining regeneration efficiency across both *Cucumis* and *Cucurbita* species. Their study demonstrated that cotyledon and mesophyll tissues often yield higher protoplast viability and regenerative capacity

compared to other tissues. Moreover, they showed that some genotypes possess a strong embryogenic potential, whereas others exhibit limited or no callus formation under identical conditions. Jia and Fu [67] were among the first to successfully achieve plant regeneration through somatic embryogenesis from protoplasts isolated from cotyledon tissue in cucumber. Their protocol involved a culture medium enriched with auxins (such as 2,4-D) and cytokinins (like KIN), which promoted initial cell wall formation, subsequent callus induction, and finally embryoid development. Moreover, reactive oxygen and nitrogen species also play critical roles in protoplast viability and regeneration capacity. Petrivalsky and Vanícková [68] demonstrated that appropriate management of oxidative stress during early protoplast culture can significantly enhance regeneration outcomes. This pioneering work established a foundational platform for further advancements in cucumber protoplast regeneration systems.

At the molecular level, Cápal and Ondřej [63] investigated the gene expression profiles and chromatin changes in cucumber protoplast cultures. They reported that dedifferentiation and cell cycle re-entry were associated with alterations in chromatin structure, particularly histone modifications and chromatin relaxation. These changes facilitate the transition of isolated protoplasts back to a pluripotent state, which is essential for initiating cell division and morphogenesis. Supporting this, Moricova and Ondrej [69] demonstrated that protoplast culture leads to significant changes in DNA methylation (5-mC) and hydroxymethylation (5-hmC) levels. Their findings suggest that epigenetic reprogramming plays a crucial role in successful regeneration, as these modifications are likely involved in reactivating genes required for cell proliferation and differentiation. High levels of 5-hmC, in particular, were linked to the early stages of organogenesis and could serve as epigenetic markers of regenerative potential.

Protoplast fusion has emerged as an effective method to overcome sexual incompatibility barriers in cucumber breeding by enabling somatic hybridization between *Cucumis* species. In their pioneering work, Fellner and Lebeda [70] successfully isolated and fused protoplasts derived from *Cucumis sativus* and *Cucumis melo*, two species that are sexually incompatible via conventional crossbreeding. Protoplasts were enzymatically isolated from leaf mesophyll tissues and fused using polyethylene glycol (PEG)-mediated fusion, a common technique that induces membrane fusion and cytoplasmic mixing. The fused cells were cultured on optimized media to regenerate hybrid calli. Although complete plant regeneration was not reported in this initial study, the formation of heterokaryons and hybrid cell colonies demonstrated that interspecific gene transfer was feasible, offering potential for the introgression of melon traits (e.g., disease resistance or fruit quality) into cucumber. Skálová and Navrátilová [16] explored haploid and mixoploid cucumber protoplasts, focusing on their isolation, fusion behavior, and chromosomal constitution. The researchers used a highly purified enzymatic mixture (typically cellulase and macerozyme) to isolate protoplasts from in vitro-grown cucumber tissues, followed by fluorescent staining and flow cytometry to determine ploidy levels post-fusion. PEG-mediated fusion was again used to generate somatic hybrids, and the resulting mixoploid protoplasts were assessed for cell division and potential to regenerate. Their analysis showed successful fusion and partial chromosome doubling, suggesting that this method could be used to develop polyploid lines or somatic hybrids with novel trait combinations. Importantly, they demonstrated that ploidy manipulation via fusion can be monitored and stabilized during early culture phases critical for downstream regeneration and breeding applications.

3. Somaclonal variation in cucumber

Somaclonal variation refers to the genetic or epigenetic changes that occur during *in vitro* plant regeneration, often from callus or suspension cultures. It can manifest as chromosomal mutations, gene sequence alterations, or methylation changes [71]. In cucumber, this phenomenon presents both a challenge and an opportunity, posing potential risks for clonal fidelity during micropropagation while also offering a valuable source of new genetic diversity for breeding. These variations, which can be genetic or epigenetic, often originate from callus or suspension cultures and may appear as chromosomal abnormalities, gene mutations, transposon activation, or DNA methylation changes. Several studies have demonstrated that explant type, physiological status, culture conditions and duration, and especially medium composition including PGRs significantly influence the level and type of somaclonal changes [12, 72]. To exploit this variation effectively, precise culture protocols are essential. This includes minimizing the number of subculture cycles since prolonged *in vitro* exposure correlates with higher variation rates avoiding excessive callus proliferation phases and carefully regulating the concentrations of PGRs [73].

In cucumber, somatic embryogenesis and callus-based regeneration are widely used in tissue culture protocols, and these methods have been linked with considerable variation among regenerants. Custers and Zijlstra [74] observed that although R_1 somaclones displayed morphological variability, most aberrations were transient and eliminated through sexual reproduction, with only a few stable variants persisting into the R_2 generation. However, modern molecular tools such as next-generation sequencing have revealed extensive underlying genomic variation in cucumber somaclones including thousands of single nucleotide polymorphisms (SNPs) and structural variants despite relatively subtle phenotypic manifestations [10, 75].

Pawelkowicz and Skarzynska [10] provided deep transcriptomic insights by comparing three somaclonal cucumber lines (S1–S3), derived from the same inbred line (Borszczagowski B10) but regenerated via different *in vitro* pathways direct organogenesis, callus-mediated regeneration, and embryogenic suspension culture. Each somaclonal line displayed distinct morphological and transcriptional profiles. Hundreds of differentially expressed genes (DEGs) were identified, related to stress response, metabolic processes, DNA/RNA binding, and transcriptional regulation. Notably, the DEGs overlapped partially across lines, indicating the existence of conserved molecular targets responsive to culture-induced stress. Moreover, several DEGs were associated with SNPs, supporting the role of both genetic and epigenetic mechanisms in driving somaclonal variation. Earlier studies corroborate these findings. Plader and Malepszy [76] and Ladyzynski and Burza [77] demonstrated that cytokinin-rich media, especially those containing BAP, increased the frequency of chromosomal abnormalities and ploidy shifts in cucumber regenerants. Bartoszewski and Havey [78] further reported alterations in organelle genomes in somaclonal lines, underscoring the broad impact of culture conditions on genomic integrity.

While somaclonal variation introduces uncertainty in tissue culture applications where genetic fidelity is essential (e.g., micropropagation or germplasm conservation), it can be deliberately harnessed as a tool for genetic improvement in cucumber breeding. Controlled induction of somaclonal variation has been shown to generate novel traits such as altered fruit shape and size, enhanced tolerance to abiotic stressors (e.g., salinity, drought), and increased disease resistance—all without the need for transgenic modification [12]. These variants serve as a valuable resource in breeding programs, particularly for hybrid development and functional genomics studies.

Unlike conventional mutation breeding or genetic engineering, somaclonal variation offers a non-genetically modified organism (non-GMO) alternative that may be more readily accepted in regions with regulatory constraints or consumer resistance.

4. Transgenic approaches and CRISPR/Cas9 genome editing in cucumber

Cucumber is an essential member of the Cucurbitaceae family, serving as a model for studying sex determination, phloem biology, and fruit development. Despite the availability of high-quality genomic resources, conventional breeding has limited success in developing resistant cultivars due to limited natural variation and sexual incompatibility barriers. As a result, transgenic and genome-editing strategies have gained momentum for introducing or modifying desirable traits. CRISPR/Cas9 technology in particular allows for efficient and targeted gene editing, representing a breakthrough in cucumber biotechnology.

Genetic transformation in cucumber has primarily relied on *Agrobacterium tumefaciens*-mediated protocols. Cotyledonary explants are the most frequently used starting material due to their high regenerative potential. Zhao and Qi [79] demonstrated an optimized transformation system using proximal cotyledon explants of 2-day-old seedlings. Their protocol included pre-culturing on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L ABA, followed by infection with Agrobacterium strain EHA105 in the presence of 100 μM acetosyringone. The selection and regeneration media contained kanamycin and meropenem, leading to a transformation efficiency of 4.67%. Xin and Tian [80] further improved this process by integrating a vacuum infiltration step, significantly enhancing transformation efficiency across different cucumber cultivars. They also showed that transformation success was strongly genotype-dependent, suggesting the need for tailored protocols for elite lines. Nanasato and Konagaya [81] also developed an effective system using the EHA105 strain and cotyledon explants precultured in a BAP-rich medium. Their regeneration system supported the recovery of transgenic lines at frequencies exceeding 8%. In addition, Nishibayashi and Kaneko [82] used hypocotyl explants for transformation and noted comparable results to cotyledon explants, suggesting broader options in explant source selection. In addition to BAP and ABA, various combinations of cytokinins (TDZ, KIN) and auxins (NAA, IAA) have been tested to optimize shoot induction and rooting in transgenic tissues. Ni and Li [83] successfully used such protocols to integrate the nattokinase gene into cucumber plants using CRISPR/Cas9.

CRISPR/Cas9 technology has enabled precise gene knockouts in cucumber, particularly for disease resistance. The mildew resistance locus O (MLO) gene family has been a major focus due to its negative regulatory role in powdery mildew resistance. Tek and Calis [84] and Shnaider and Elad [85] reported successful editing of CsaMLO8, which conferred strong resistance to Podosphaera xanthii without compromising plant morphology. In the viral disease context, Fidan and Calis [86] disrupted the *eIF4E* gene using CRISPR/Cas9. This gene is a known susceptibility factor for several potyviruses. Edited cucumber lines displayed resistance to WMV, ZYMV, and PRSV, and importantly, these plants maintained high fertility and morphological integrity. Chandrasekaran and Brumin [87] laid the groundwork for virus resistance via targeted genome editing by demonstrating that eIF4E mutants are broadly resistant to RNA viruses. Their work influenced further studies targeting translation initiation factors in cucurbits. Zheng and Yang [88] targeted CsNBS-LRR gene families involved in bacterial wilt resistance and reported successful gene editing with significant reductions in disease symptoms.

Beyond disease resistance, genome editing technologies have been increasingly applied in cucumber to engineer metabolic traits, enabling nutritional enhancement and the production of high-value bioactive compounds. Ni and Li [83] introduced the *nattokinase* gene into cucumber plants using CRISPR/Cas9. This not only demonstrated the crop's amenability to foreign gene insertion but also highlighted cucumber's potential as a molecular farming platform for nutraceutical and pharmaceutical production. Additionally, Mitra and Shnaider [89] used CRISPR/Cas9 to study genes involved in fruit set and development. Knockouts of candidate genes like *CsGA3ox* and *CsIAA9* revealed their regulatory roles, allowing manipulation of fruit formation pathways. Moreover, recent studies have expanded CRISPR's application to abiotic stress tolerance. Peng and Chen [90] identified *CsAKT1* as a key gene involved in salt tolerance, which was validated using CRISPR/Cas9-edited lines. The knockout mutants displayed altered potassium uptake and enhanced salt resistance. Dong and Li [91] focused on the *STAYGREEN* gene, showing its role in low-temperature tolerance. Genome-edited lines exhibited improved chlorophyll retention and better cold tolerance.

To accelerate gene function studies, Nguyen and Hoang [92] developed a hairy root transformation system using *Agrobacterium rhizogenes*. This method allows the rapid assessment of CRISPR constructs without the lengthy regeneration process. Transgenic hairy roots expressing Cas9-sgRNA cassettes successfully induced mutations in target genes within 10–14 days. Kiryushkin and Ilina [93] demonstrated hairy root-based CRISPR editing for root architecture studies. Their work provides a foundation for applying root-focused gene manipulation, particularly under stress or nutrient-deficient conditions.

5. Conclusion

In vitro culture technologies combined with modern genetic tools have transformed cucumber improvement. Using regeneration systems like organogenesis, somatic embryogenesis, haploid induction, and micropropagation, researchers have built platforms for trait introgression, rapid propagation, and genome editing. Explants such as cotyledons and hypocotyls, with optimized hormone combinations (e.g., BAP, NAA, TDZ), enable high-efficiency shoot and root regeneration, which is foundational for transformation. Advances in Agrobacterium-mediated transformation ensure stable gene integration, while CRISPR/Cas9 offers precise editing for functional genomics. Editing genes related to biotic (e.g., *MLO*, *eIF4E*) and abiotic stress (e.g., *CsAKT1*, *CsSGR*) yields lines with better resistance and adaptability. Metabolic engineering for traits such as β-carotene and vitamin C highlights cucumber's potential for functional foods. Challenges include genotype dependence, limited protocols for elite cultivars, and unclear regulatory guidelines. Future focus should be on genotype-independent systems, improved CRISPR delivery, and omics integration for multi-target editing. Overall, combining *in vitro* methods with genome editing promises to enhance cucumber breeding, thereby increasing yield, resilience, and nutritional value for global agriculture.

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