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Review Article

Tissue Culture Optimization: Maize Callus Induction and Factors Affecting the Callus Efficiency

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ABSTRACT

Maize (*Zea mays L.*), also known as the queen of cereals, is one of the most important food crops in terms of food security, the world economy, and global agriculture. The high yield, diverse climate adaptability, and extensive applications of maize in food, feed, and industrial products make it a highly demanding crop. The traditional methods of maize propagation are slow and often result in less yield with undesired traits due to large-scale variations. However, the advancements in agricultural biotechnology, particularly in plant tissue culture, have made it possible to propagate maize cultures with desired characteristics in *in-vitro* lab settings. Tissue Culture has enabled researchers to improve crops for rapid propagation, genetic modification, and production of resilient varieties. It has also enabled to development of identical cell lines, through callus induction, to control the gene pool of a specific genotype. Callus induction is the formation of an unorganized mass of cells from an explant in tissue culture settings. Maize tissue culture is performed to develop varieties of improved traits and high yields. Callus induction of maize is a complicated process because different factors influence maize callus induction, particularly the genotypes of maize and the type of explants. Different maize genotypes respond differently to the induction conditions. Some genotypes can easily be cultured while others are difficult to culture. Similarly, different types of explants respond differently to the culture conditions. Immature embryos need different concentrations of plant growth regulators (PGRs) than the leaf explants. This review focuses on the optimized process of maize callus induction in a controlled environment and the effects of different PGRs (auxins, cytokinins, etc.), and factors (explant, genotypes, pH, environmental conditions, etc.).

Keywords: Maize, Tissue Culture, Callus induction, Callus efficiency, Plant Growth Regulators, Protocol optimization

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INTRODUCTION

Maize (*Zea mays* L.), the third leading crop of the world after rice and wheat, belongs to the family *Poaceae* (Saeed, 2020). It is a paramount cereal crop in the world. Zea is a Greek word that means “sustaining life” and Mays is a word from the Taino language meaning “life-giver” (Rouf Shah *et al*., 2016). Maize has different local names around the world like Makai, Bhutta, Cholam, Agbado, Mahindi, and Jagung. It is considered a staple food in many parts of the world. Due to its high adaptability, maize can be grown in various climates. Maize is grown globally at an area of 197 M ha (Erenstein *et al*., 2022). According to FAOSTAT, the average yearly production of maize was 1036 billion tons in the last decade (Dragomir *et al*., 2022). The United States of America (USA) is the largest producer of maize as it contributes around 36.1% of the total maize production in the world (Erenstein *et al*., 2022; Jiang *et al*., 2020; Rana *et al*., 2018).

In Pakistan, maize is cultivated on 3503.5 thousand acres of land, and production of maize has increased to 8939 thousand tons (Islam *et al*., 2023). Maize is also known as the queen of cereals because it has the highest yield potential. The major maize-growing cities in Pakistan are Sahiwal, Okara, Faisalabad, Sheikhupura, Kasur, Swat, Mardan, Charsadda, and Mansehra. It is integral to sustainable agricultural practices. The nutritional content of maize generally consists of 73% starch, 10% protein, 5% oils, and the remainder of fiber, minerals, and vitamins (Eckhoff & Paulsen, 1996). Maize is generally used as food, animal feed, and raw material for various industrial products such as biofuels, sweeteners, and biodegradable plastics (Murdia *et al.*, 2016). There are various product types in which maize is processed in food industries such as snacks, corn flour, cereals, starch, etc. (Serna-Saldivar, 2023). In agricultural biotechnology, maize has been widely studied, from tissue culture to genetic transformation, to increase yield, enhance resistance, and produce more varieties suitable for different climates. Tissue culture and Genetic transformation played a vital role in the enhancement of maize. Tissue culture techniques helped to rapidly multiply the number of maize plants and develop disease-free and uniform varieties (Long *et al*., 2022). Genetic Transformation allowed biotechnologists to introduce new techniques for better breeding traits. Furthermore, CRISPR-based gene editing enabled the researchers to manipulate the genome which led to the development of new maize varieties that are more resistant and high-yielding (D. Dai *et al*., 2021). By improving the genome of maize, researchers have made significant progress in maize crops for economic purposes.

TISSUE CULTURE TECHNIQUE

Plant tissue culture is an in-vitro technique of aseptically culturing the cells, tissues, organs, and their components under controlled physical and chemical conditions (Phillips & Garda, 2019). Maize has been successfully tissue cultured in in-vitro lab settings and many labs around the globe have developed a defined propagation protocol (Ahsan *et al.*, 2000). The general outline of plant tissue culture is given in Figure 1. Below is a detailed discussion of the maize tissue culturing process:

Explant Selection

Explants are tiny pieces of totipotent tissue from plants used in tissue culture. They serve as the starting point for in-vitro culture, produced using certain medium compositions under closely controlled conditions and aseptic procedures (Micheli *et al.*, 2020). Various possible maize explants, including buds, mature and immature embryos, leaves, stems, roots, meristems, flowers, and seeds, can be used for tissue culture (Illg, 1991). An appropriate explant is critical in tissue culture since it affects the entire process. The selection of plants depends on several variables. Compared to immature embryos or tissues, mature embryos or stem cells are less receptive to regeneration in tissue culture (Wenzel *et al.*, 1985). It is important to collect the explants at the appropriate stage of development. For best results in culture, immature embryos, for instance, should be retrieved 10–15 days after pollination (Rakshit *et al.*, 2010). The explant has to be free of pests and illnesses. This will ensure healthy culture development and stop contamination. The explant should be of proper size. Explants that are extremely big or little could not be at the right developing stage, thus they wouldn't be useful. Those Explants that have been successfully cultivated in the past and have been used in literature should be given careful consideration. From all of the explants available, immature embryos are widely used in tissue culture because of their high regenerative capacity and responsiveness to tissue culture conditions (Vikrant and Rashid, 2001). Also, immature embryos showcase high efficiency in genetic transformation, making them an ideal explant for maize tissue culture (Özgen *et al.*, 1996).

Media Preparation and Sterilization

Culture media is a nutrient-rich solution that is used in tissue culture to grow and develop plant tissues and cells. Vital minerals, vitamins, and other growth elements are provided via a culture medium (Huang and Murashige, 1977). Murashige and Skoog (MS) medium is the best culture medium that is appropriate for the tissue culture of maize since it is full of all the necessary vitamins and nutrients. Determine the volumes of each ingredient needed to prepare media at the precise concentrations. If you wish to produce a solid medium, add agar at the end (Norstog K, 1963). To sterilize the medium, autoclave it after adding all the ingredients. The media is transferred to culture vessels or petri plates following autoclaving. Contamination is a major issue in in-vitro plant tissue culture, and this causes hindrance in the process of micropropagation. For that reason, we have to follow the aseptic parameters which help to minimize the contamination. Sterilization includes many chemical decontaminants named as Ethanol (C_2H_5OH), Sodium Hypochlorite (NaOCl), Mercuric Chloride ($HgCl_2$), and Tween 20, etc. (Misra and Misra, 2012). These chemicals are used in specific concentrations depending on the surface or explants to be decontaminated.

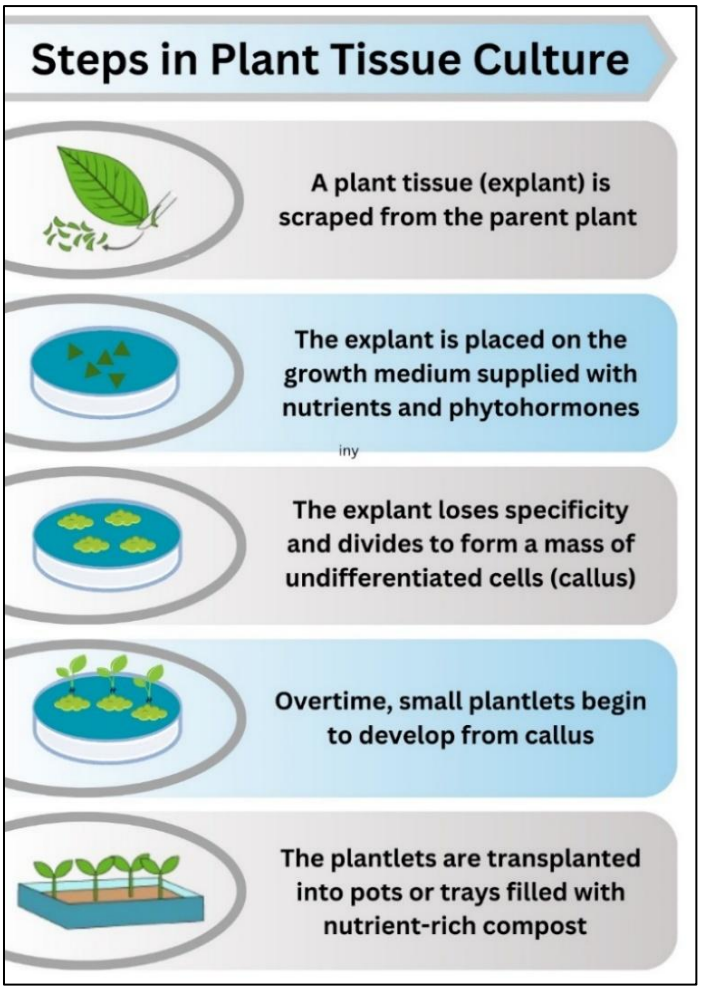

Figure 1: General steps in plant tissue culture.

Sodium Hypochlorite (NaOCl) and ethanol are frequently used in maize tissue culture to sterilize the explants' surfaces. As explants are usually covered with dirt or mud, they should be thoroughly washed at least twice in the first sterilizing procedure using distilled water. The subsequent phase includes immersing the explants in a container or reagent bottle containing 70% ethanol for five to seven minutes. Sterilization of explants is successfully facilitated by this method. The final stage of sterilizing is to soak the explants in a 10% to 15% sodium hypochlorite (NaOCl) solution for 10-15 minutes (Green *et al.*, 1974). The explants are thoroughly rinsed with water to eliminate any residual decontaminants after every sterilization treatment (Gamborg, 1991). Culture media are the most important component of tissue culture because all of the propagation depends on it. Culture media are the best place for the growth of biological contaminants. The prepared culture media are autoclaved at 121℃ and 15 psi pressure for 15-20 minutes (Burger, 2022). The working space (laminar flow hood) and the tools used for tissue culturing can contain a lot of biological contaminants. To overcome this contamination, all the suitable tools are autoclaved to decontaminate them. The surfaces are wiped with 70% ethanol, killing most of the microbes. Also, the laminar hood can be treated with UV light for 12-15 minutes which is a promising way to sterilize the surface (Sathyanarayana and Varghese, 2007).

Inoculation and Incubation

Inoculation refers to the culture of selected explants to the media containing petri plates or culture vessels in sterile conditions (under a laminar flow hood). The cultured plates are then sealed with parafilm to prevent contamination (Bhoite and Palshikar, 2014). Cultured explants are kept under carefully monitored conditions of temperature, light, and humidity in a growth room or growth chamber. The optimal conditions for maize tissue culture are 25–28°C, 70–80% relative humidity, and a 16-hour light/8-hour dark photoperiod (Guruprasad *et al.*, 2016). Plantlet regeneration with roots and branches is followed by sub-culturing, which is the process of transferring developing tissues to various mediums to stimulate further development. When plantlets are fully developed, they go through a phase called acclimatization, in which they gradually get used to their surroundings. After that, the plants are transplanted into the field and cared for there until they grow sturdy roots. Many procedures are required to transform vitro-grown tissues into live, self-sufficient plants (da Silva *et al.*, 2017).

CALLUS INDUCTION IN MAIZE

Maize callus induction refers to the process of formation of a mass of unorganized cells in the controlled culture conditions through the influence of different plant growth regulators, particularly auxins, that stimulate cell division but prevent the normal differentiation of cells into organized maize tissues. A callus contains totipotent cells that can be differentiated into particular tissues or even whole plants depending upon the desired experimental results. This is an important process in plant tissue culture as from a healthy callus, we can perform multiple tasks like genetic transformation, cell line generation, and tissue manipulations. In plant tissue culture, callus induction is a very crucial step in the development of new varieties based on genetic manipulations and genetic transformation (Fehér, 2019). It can also be called the intermediate stage of the tissue culture process headed toward a particular type of experiment such as cell line generation of genetic transformation (Fehér, 2019). The significance and purpose of tissue culture is described in a later section.

Purpose and Significance of Callus Induction:

Regeneration

The most basic purpose of callus induction in maize tissue culture is to develop an intermediate product for further plant regeneration. Callus has very significant applications, as it can be differentiated and regenerated into any plant tissue, depending upon the culture conditions (Fehér, 2019). For example, the callus developed from an immature maize embryo can be regenerated into shoots, roots, or even whole plants with the help of different growth regulators. This process enables the researchers to propagate plants from a small tissue sample allowing for the regeneration of multiple plants with desired traits. Although, the concentrations of PGRs play a key role in determining the type differentiation.

Somatic embryogenesis

Somatic embryogenesis is a pivotal process in maize tissue culture. In this process, the non-reproductive cells (somatic cells) are induced to develop into structures like zygotic embryos and are named somatic embryos (Martínez *et al*., 2024). These somatic embryos follow the same developmental path as naturally occurring zygotic embryos but arise from somatic tissues rather than gametes. These somatic embryos can be germinated under suitable conditions directly after the conversion of callus tissues into plant embryos (Zhang *et al*., 2021). The major benefit of somatic embryogenesis is that it can form large quantities of genetically identical plants from a single explant. These identical plants are called clones. This ability of somatic embryogenesis makes it an advantageous process in plant breeding programs. This technique accelerates the breeding process, which generates new maize varieties.

Genetic Transformation

Genetic transformation is an advanced process of integrating a foreign gene into an organism's genome through different techniques to create transgenic plants with new traits and this technique is widely used in maize biotechnology (Keshavareddy *et al*.,2018). The new traits could be disease resistance, increased nutritional value, herbicide tolerance, etc. The most common techniques for genetic transformation in plants are Agrobacterium-mediated transformation or biolistics (Anjanappa *et al*., n.d.; Babich *et al*., n.d.; Keshavareddy *et al*., 2018). These methods easily deliver the desired genes into plant cells. However, the efficiency of both of these methods differs from each other. As callus cells can regenerate into whole plants they can be easily transformed and subsequently regenerated into transgenic plants (Tiwari *et al*., 2015). Once the new gene has been transferred to the maize callus, it can be induced to form shoots and roots which ultimately regenerate into a whole new transgenic maize plant. Thus, callus induction is a very important step in maize genetic transformation.

Mass Propagation

As described earlier, a callus is a mass of undifferentiated cells that can be induced from a single explant i.e. immature embryo, root tips, etc. So, a callus from a single explant conserves genetic resources in all the undifferentiated cells. Thus, by maintaining these callus cultures, a very large number of similar plants can be produced from a single explant, which is commercially very important for maize production all over the world (Hildebrandt and Riker, 1953). This method is very beneficial for the rapid multiplication of maize plants with desirable traits. These traits could be high yield, pest resistance, drought tolerance, etc. By generating cell lines in suspension solutions, callus cultures can provide long-term preservation of particular maize genotypes. This can be very important for preserving the endangered maize varieties.

Somaclonal Variations

Somaclonal variations are the genetic and epigenetic changes that occur during the process of plant tissue culture. There can be many reasons for these types of changes but these variations are beneficial, sometimes, when culture uniformity is not required. Somaclonal variations can induce unique traits of stress tolerance, disease resistance, or crop improvement (Krishna *et al*., 2016). This leads to an ease in natural selection in plant breeding programs.

Experimental Flexibility

Callus cultures are a valuable tool in maize tissue culture because the *in-vitro*-maintained callus cultures can be used to study the cell response to various environmental conditions, media compositions, and hormonal concentrations. Every single environmental element can affect the health of the callus. For example, light intensity, humidity, and temperature. The media compositions also play a vital role in callus induction. A slight change in the concentrations of media components can cause the lysis of the callus or can play an inhibitory role in callus induction. So, callus cultures allow us to study the effects of different concentrations of different elements required for healthy plant growth. This also helps to establish potential protocols for plant growth and development. Callus systems can be used as model systems to study plant biology, including gene expression, metabolic pathways, stress response, and the mechanism underlying tissue differentiation. The insights gained from these studies can be helpful in plant breeding programs to develop more resilient maize varieties.

Factors Affecting Callus Induction in Maize

In-vitro callus induction is not a straightforward process, as described earlier, there are many factors involved that influence callus induction, and they play a vital role in successful callus induction. Even a minimal variation in any of these factors can affect the callus health. These factors are described as:

Genotypes of Explants

The genotype of the maize explant is the most critical factor for successful callus induction. Efficient callus induction in maize highly depends on the genotypes of the maize explants which can affect the callus formation, callus quality, and regeneration ability of the cultured explants. The explant genotypes play a major role in the process of callus induction, and these genotypes react differentially to the conditions of tissue culture. This is a result of the explants' differing genetic composition. The response of maize to extrinsic stimuli for callus induction, such as plant growth regulators (PGR) and the culture environment, is determined by its genetic makeup (Ye *et al*., 2022). Research indicates that some genotypes of maize have more efficacy in inducing embryogenic callus, increasing the likelihood of the plant's regeneration into a whole. Further studies revealed that the genetic information of those callus-producing maize inbred lines is responsible for the difference as these inbred genotypes behave differently in tissue culture environments. Specific regions on the chromosomes enable specific genotypes to show response in tissue culture. Chromosome number 3 was found to be associated with the embryogenic callus induction of the A188 inbred line of maize (McFarland *et al*., 2023). Some genotypes are highly suitable for the *in-vitro* tissue culture process and some are not. As found in the study, the NM74C, NM81A, and NM5883 inbred lines showed more response to calli formation than the NM5884 which showed no response (Muppala *et al*., 2020). However, this is not limited to maize only because some other crops, like rice and peanuts, have the same genotype dependencies. Table 1 shows the different genotypes of maize and their different responses to callus culture conditions.

Callus induction involves a complex process of physiological and biological pathways which are controlled by multiple genes. Studies suggest that callus induction is a polygenic process as the specific genotypes that induce efficient and high-frequency callus are influenced by multiple genes and every gene contributes a particular effect to the induction and maintenance (Du *et al*., 2019). Quantitative Trait Loci (QTL) analysis and Genome-wide association studies have identified specific genetic loci associated with improved callus induction and regeneration rates in maize. The QTL analysis of 126 recombinant inbred lines (RILs) of maize inbred H99 (high callus response) by inbred Mo17 (low callus response) observed 11 bins (8 with main effect and 3 with epistatic effect) on 8 chromosomes (Krakowsky *et al*., 2006). Polygenic influence has also been studied in other plants like Populus *trichocarpa*, *Elaeis guineensis,* and *Oryza sativa* (Htwe *et al*., 2024; Tuskan *et al*., 2018; Z. Zhang *et al*., 2019). Some studies have revealed the role of specific genes in the hormone-signaling pathways of auxin and cytokinin. These hormones are essential for cell division and differentiation. Thus, multiple genes are involved in determining the genotype's response to tissue culture conditions.

Media Compositions

The second most influential factor in callus induction is the media composition in which the explant is placed. Media consists of many components such as basal medium, carbon source, and vitamins and each of these components plays a vital role in maize callus induction. Basal media is the base of *in-vitro* tissue culture, and this media is usually used for tissue culture experiments. It contains micronutrients, macronutrients, vitamins, inorganic salts, sucrose or carbon sources, and sometimes amino acids. The most commonly used basal media for maize are Murashige and Skoog (MS) medium, N6 medium, Linsmaier and Skoog (LS) medium (Gamborg *et al*., 1976; Greenway *et al*., 2012; Phillips and Garda, 2019). All of these basal media are used to induce callus in tissue culture conditions but the efficiency of all of these media is different. The most widely used basal medium with the highest efficiency rate is the Murashige and Skoog (MS) medium due to its comprehensive composition of micronutrients and macronutrients (Li, 2024).

Table 1. Response to callus induction of multiple maize genotypes.

Sr.	Genotype	Response to Callus Induction	References
1.	K-25	+++	(Monalisha, 2018)
2.	L4212	++	(Monalisha, 2018)
3.	BVM-2	+	(Monalisha, 2018)
4.	COH(M) 5	+++	(Malini *et al*., 2018)
5.	UMI 757	+	(Malini *et al*., 2018)
6.	UMI 615	+	(Malini *et al*., 2018)
7.	UMI 112	++	(Malini *et al*., 2018)
8.	UMI285	+	(Malini *et al*., 2018)
9.	CO 1	+	(Malini *et al*., 2018)
10.	Burak	+++	(Tanur and Mustafa, 2017)
11.	Safak	+++	(Tanur and Mustafa, 2017)
12.	Xianzao	+++	(Hong-mei *et al*., 2004)
13.	18-599 hong	++	(Hong-mei *et al*., 2004)
14.	18-599 bai	++	(Hong-mei *et al*., 2004)
15.	Ji 53	+++	(Hong-mei *et al*., 2004)
16.	3189/4380	+++	(Hong-mei *et al*., 2004)
17.	4380/Shanzong 5	+++	(Hong-mei *et al*., 2004)
18.	8103	+++	(Hong-mei *et al*., 2004)
19.	Reid group	+	(Hong-mei *et al*., 2004)
20.	Tangsipingtou group	+++	(Hong-mei *et al*., 2004)

Note: (+++) shows the highest response of genotype to callus. (++) shows the moderate response of genotype to callus. (+) shows the lowest response to maize callus induction.

The high levels of nitrogen in the form of nitrates are essential for callus induction and show promising results (Morshed *et al*., 2016). This media is not only recommended for callus induction of maize but also for plant regeneration. Other than maize, this media is also suitable for a wide range of different plant species. The best concentration for full-strength MS media is 4.34 g/L (Anami *et al*., 2010; Malini *et al*., 2015). This is the ideal concentration of MS medium and studies have proved that this concentration is suitable for almost all of the tissue culture experiments. Although, this medium can be used as half-strength with a concentration of 2.2 g/L for specific genotypes of maize. N6 nutrient medium was first developed for rice anther culture but now it has been formulated for maize tissue culture as well (Nicolas *et al*., 2021; Fu *et al*., 2011). The high nitrogen levels provided by glycine and casein hydrolysate in the N6 basal medium significantly benefit the callus induction process (Kaushal *et al*., 2014). N6 medium is sometimes preferred over MS medium because of its nutrient composition as it better supports the embryogenic callus formation in maize. Studies showed the best concentration of N6 basal medium is 2-4g/L (Fu *et al*., 2011; Pathi *et al*., 2013). Linsmaier and Skoog (LS) Medium can also be used for callus induction in certain genotypes of maize. The composition of LS media is slightly different from N6 and MS medium and depends on the specific tissue culture needs (Neondo, 2014). Callus development is also influenced by vitamin and carbon source concentrations; however, these may be controlled and may be the same for both basal media. While maltose and glucose can be added as carbon sources, sucrose (2–3% or 20–30 g/L) is the most advised carbon source (Nickell, 1970; J. Wang, 1974; H. Karstens, 1960). Greater sucrose concentrations have the potential to create non-embryogenic callus, which is not advised because it is not suited for future regeneration.

To induce a healthy and viable callus, vitamins and amino acids like thiamine, nicotinic acid, pyridoxine, glycine, and casein hydrolysate are most frequently included as supplements to callus induction medium (Rodríguez G. *et al*., 1998; Sudheer *et al*., 2022). These substances support rapid cell division, proliferation, callus formation, and cell growth and development. Furthermore, the usage of various media is contingent on the desired outcome of the experiment. Thus, the experiment dictates how these mediums are employed. However, precise and ideal medium concentrations are crucial for strong and efficient callus induction and tissue culture protocol development. The outcomes can be affected by even a small shift in concentration.

Plant Growth Regulators (PGRs)

The concentration of Plant Growth Regulators (PGRs) is a critical component that affects callus induction efficiency. These are basically the substances that play key roles in plant development and morphogenesis. These substances are required

in low concentrations, but they are essential for plant growth (Beyl, 2011). An undifferentiated mass of cells is formed from plant tissues in callus induction, and the amount and type of PGRs utilized significantly impact how well this induction works. The concentration of PGRs directly influences the cellular response of maize tissues. PGRs like auxins and cytokinins act as signaling chemicals, controlling cell division, differentiation, and growth (Farman *et al*., 2019; Sidik *et al*., 2024; Small and Degenhardt, 2018). These hormones can efficiently promote the production of callus tissue when their concentration is optimum. On the other hand, unsuitable concentrations may result in negative outcomes like delayed growth or cell death. For example, high amounts of auxin might result in tissue necrosis, whilst low concentrations might not allow for significant cell division (Gaba, 2005). Plant tissues react differently to PGR concentrations such as callus tissues, leaf explants, and immature embryos. The distinct physiological characteristics of every tissue type affect how sensitive it is to PGRs. As a result, the ideal concentrations need to be adjusted for the particular tissue type being grown. For example, immature embryos could need different quantities of PGRs than leaf explants to induce maximal callus induction. The genetic makeup of maize explant can significantly affect its sensitivity to various PGR concentrations. Different maize genotypes exhibit varying responses to PGRs, necessitating an understanding of optimal concentrations for specific genotypes. Research has shown that certain inbred lines may respond better to higher concentrations of cytokinins, while others may require increased auxin levels for optimal callus induction. This genotypic variation underscores the importance of customizing PGR concentrations to enhance the efficiency of tissue culture techniques.

There are a variety of auxins**,** in our case 2,4-D (2,4-dichlorophenoxyacetic acid) is one such important hormone and it is pivotal to cellular division as well as elongation. It is also necessary for the phenomenon called root formation all across different plant tissues that aptly help them grow and develop. The efficient induction of friable callus, a collection of cells that have the potential to differentiate into diverse organ structures among higher plants, occurs in higher auxin concentrations (Ikeuchi *et al*., 2013). It appears that high concentrations of auxins are typically required to effectively and rapidly induce callus tissue formation (Shin and Seo, 2018; Ma *et al*., 2020). It is reported by comprehensive investigations that keeping the concentration of 2,4-D in a range like this (1-4 mg/L) would be more beneficial to callus induction and supporting those essential tissue aggregates proliferate (Çabuk and Özgen, 2016; Jiao *et al*., 2020; Mayerni *et al*., 2020; Naqvi *et al*., 2002; Pathi *et al*., 2013; Rahayu *et al*., 2016). However, it is important to note that when the auxin levels go too high, there will be deleterious effects associated with tissue necrosis (the death of a portion of all cells in an organ or structure), which may severely inhibit plant recovery from certain environmental incidents. Therefore, it is critical to determine the best combination of auxins leading to robust and viable callus tissue generation on which subsequent plant regeneration can build up.

Cytokinins**,** including the most important compounds like Kinetin and BAP (Benzylaminopurine), are essentially critical for activating cell division and shoot organogenesis in plant tissues contributing to an irreplaceable part of the entire course of growth and development process during a plant lifecycle (Mok, 2019). Surely, the concentration of cytokinins also must be balanced well with those of auxins to achieve (or enhance) induction of callus (Agha *et al*., 2022). The specific balance between the concentrations of auxins and cytokinins is highly dependent on the specific genotype of the plant. Some genotypes of maize are independent of this balance as they have shown callus induction without the addition of cytokinin in culture media (Astutik *et al*., 2022). Some plant genotypes don't have the limitations of higher auxin to cytokinin ratio as they require 1:1 of both as investigated in date palm (Solangi *et al*., 2020). There are some protocols reported that induced callus with high cytokinin to auxin ratios (Gerszberg *et al*., 2015a). Normally, the levels of cytokinins (kinetin) used are from 0.5 to 2 mg/L in maize, although it can vary with the specific genotype under study (F. Ali, *et al*., 2014; Gerszberg *et al*., 2015a; Naqvi *et al*., 2002; Pathi *et al*., 2013; ZHAO *et al*., 2008). Balanced auxins to cytokinins provide the required ratio for callus dedifferentiation and subsequent regeneration of organs with plantlet formation. Besides regular auxins and cytokinins, IAA and Zeatin in particular several other plant growth regulators (PGRs) could have a relevant impact on the process such as gibberellins or abscisic acid (Bourquin and Pilet, 1990). These PGRs may not be as widely involved in the initial stages of callus induction as earlier listed, however, they can be crucial for the later stages. Gibberellins, known for stimulating the elongation and expansion of plant tissues are very characteristic in several development stages while abscisic acid is vital to mediate responses to external stresses facilitating the appropriate disposition of diverse structures. Although gibberellins and abscisic acid are believed to have fewer dominant roles relative to auxins and cytokinins, these hormones can contribute greatly towards the overall health (vigor) of callus tissue by maintaining its strength when it comes to packing the tissues with all of their element requirements necessary for furthering division (Emons *et al*., 1993; Ouyang *et al*., 2024; Rai *et al*., 2011). The best hormonal combinations that attain the highest efficiency in callus induction can display quite important differences and discrepancies when studied along different genotypes of *Zea mays*. Different genotypes of explants require specific combinations of multiple PGRs for efficient callus induction. Table 2 shows the efficient combinations of PGRs for callus induction in reported plants.

Table 2: Balanced Combinations of different PGRs for efficient callus induction in different plants.

Plant Names	Explants	Media	Optimum Concentrations of Different PGRs for Callus Induction (mg/l)					Callus Efficiency	References
			Auxins			Cytokinins			
			2,4 D	NAA	IAA	Kinetin	BAP		
Maize	Immature Embryos	MS	1.5	--	--	0.3	--	99.10%	(Malini *et al*., 2018)
	Stem	MS	2.0	--	--	--	--	100%	(Astutik *et al*., 2022)
	Roots	MS	1.0	--	--	--	--	100%	(Astutik *et al*., 2022)
	Mature Embryos	N6	2.0	--	--	--	0.2	97.6%	(Wei and Huang, 2004)
Sugarcane	Shoots	MS	3.0	--	--	--	--	100%	(Ather *et al*., 2009)
	Meristems	MS	2.5	--	--	-	--	100%	(Behera and Sahoo, 2009)
	Leaf Whorls	MS	4.0	--	--	--	--	85%	(Patel *et al*., 2013)
Mango	Nucellar Tissues	MS	1.0	--	--	--	0.25	32.1 %	(Khair *et al*., 2016)
	Epicotyl Segments	MS	2.0	--	--	--	0.5	90.3 %	(Kishor, 1999)
Wheat	Immature Embryos	MS	2.0	--	--	--	--	93.3%	(Özgen *et al*., 1998)
	Mature Embryos	MS	8.0	--	--	--	--	98.3%	(Özgen *et al*., 1998)
	Leaf Basal Parts	MS	2.0	--	--	--	--	96%	(Kopertekh, 2003)
Mung bean	Cotyledons	MS	--	2.5	--	--	1.0	90%	(Khatun *et al*., 2008)
	Hypocotyls	MS	2.0	--	--	1.0	--	100%	(Rao *et al*., 2005)
	Leaves	MS	2.0	--	--	--	0.5	100%	(Avenido and Hautea, 1990)
Cotton	Hypocotyl	MS	0.1	--	--	0.5	--	90%	(Michel *et al*., 2008)
	Immature Embryos	MS	0.5	--	--	--	2.0	85.6%	(Kamal, 2011)
Cajanus	Epicotyl	MS	--	--	1.0	0.9	--	95%	(Prabhakaran *et al*., 2011)
Banana	Immature Flowers	MS	2.0	--	--	--	5.0	79.17%	(Ardhani *et al*., 2024)
	Sword Suckers	MS	2.0	0.5	--	--	--	70%	(Kumar *et al*., 2019)
Orange	Nucellus Tissues	MS	5.0	--	--	--	--	90%	(Hussain *et al*., 2016)
	leaf explants	MS	0.5	0.5	--	--	0.5	99.66%	(Mumtaz *et al*., 2015)
	Mature Seeds	MS	3.0	--	--	--	--	86.7%	(M. Hasan *et al*., 2019)

Coffee	leaf explants	MS	2.0	--	--	--	--	100%	(Setiawan *et al*., 2020)
Cucumber	Leaf Disc	MS	2.0	--	--	--	--	91.50%	(Sultana *et al*., 2021)
	Stem Explants	MS	--	1.0	--	--	0.5	89.0%	(Jesmin and Mian, 2016)
Rice	Mature Seeds	MS	3.0	--	--	--	--	82.0%	(Mostafiz and Wagiran, 2018)
	Anther explants	N6	1.0	3.0	--	--	--	80.45	(Rahman *et al*., 2021)
Tomato	Cotyledons	MS	--	--	0.1	--	2.0	86.6%	(Arulananthu *et al*., 2019)
	leaf explants	MS	4.0	--	--	--	0.5	90%	(Jan *et al*., 2015)
	Hypocotyls	MS	--	0.1	--	--	1.0	93.3%	(Durrani *et al*., 2017)
Tobacco	Leaf Segments	MS	--	2.0	--	0.2	--	100%	(Husin *et al*., 2005)
Apple	leaf explants	MS	--	0.5	--	--	1.0	86.6%	(R. S. Kumar *et al*., 2016)
Soybean	Cotyledons	MS	3.3	2.7	--	--	--	100%	(Joyner *et al*., 2014)
Alfalfa	Hypocotyls	MS	--	--	0.5	--	--	73%	(Shujie *et al*., 2005)
Common Bean	Epicotyls	MS	--	--	0.5	--	1.0	91%	(Kanchiswamy and Maffei, 2008)
Pineapple	Leaf Base Explants	MS	2.0	--	--	--	2.0	95%	(Amin *et al*., 2005)
Barley	Mature Seeds	MS	3.0	--	--	--	--	100%	(Al-ajlouni *et al*., 2012)
	Immature Embryos	MS	3.0	--	--	--	--	91.33%	(Hussein *et al*., 2004)
Peanut	Cotyledons	MS	--	--	3.0	--	1.0	72.8%	(Palanivel *et al*., 2002)
	Immature Leaf	MS	--	3.0	--	0.5	--	96.3%	(Venkatachalam *et al*., 1996)
Pea	Leaf Segments	MS	--	0.5	--	1.0	--	89%	(Jacobsen and Kysely, 1984)
	Embryo Axis	MS	--	2.0	--	--	2.0	100%	(Natali and Cavallini, 1987)
Chickpea	Immature Cotyledons	MS	--	2.0	--	--	1.0	90%	(Anju *et al*., 2005)
	Seed Explants	MS	2.0	--	--	--	2.0	85%	(Mansur *et al*., 2018)
	Embryo Axes	MS	3.0	--	--	--	--	82%	(Shukla *et al*., 2015)
	Cotyledon Explants	MS	4.0	--	--	--	1.0	97%	(Khan *et al*., 2011)
Sweet Potato	Leaf Explant	MS	0.5	--	--	0.5	--	90%	(Kamal *et al*., 2015)
Tea	Leaf Segments	MS	--	3.0	--	--	2.0	72.2%	(Seran *et al*., 2007)

	Hypocotyl Explants	MS	--	0.1	--	--	2.0	95.19%	(Jin *et al*., 2024)
Aloe vera	Shoot Tips	MS	1.0	0.2	--	--	--	90%	(Saggoo and Kaur, 2010)
	Leaf Explants	MS	2.0	--	--	2.5	--	100%	(Singh *et al*., 2020)
Pomegranate	Leaf Explants	MS	--	0.4	--	--	1.0	100%	(Bonyanpour and Khosh-Khui, 2013)
	Stem Explants	MS	--	1.0	--	--	1.0	72%	(Wang *et al*., 2023)
Strawberry	Runner Explants	MS	1.5	--	--	--	0.5	95.75%	(Dawa *et al*., 2017)
Papaya	Immature Embryos	MS	3.0	--	--	--	--	83.3%	(Rajesh *et al*., 2020)
Onion	Cotyledons	MS	2.0	--	--	--	--	97.14%	(Keighobadi *et al*., 2020)
	Shoot Apex Explants	MS	4.0	--	--	--	--	85.3%	(Keighobadi *et al*., 2020)
Neem	Immature Flowers	MS	1.0	0.2	--	--	1.0	78%	(Rafiq and Dahot, 2010)
	Unfertilized Ovaries	MS	0.2	--	--	--	1.1	100%	(Srivastava *et al*., 2009)
Moringa	Zygotic Embryos	MS	--	2.0	--	--	--	95.8%	(Devendra *et al*., 2012)
Rose	Leaf Segments	MS	2.5	--	--	--	--	96%	(Rahman *et al*., 2023)
	Nodal Segments	MS	4.0	--	--	--	--	90%	(Afrin *et al*., 2022)
Date palm	Mature Embryos	MS	--	1.5	--	--	1.0	100%	(Shahrour *et al*., 2024)
Rubber tree	Anther Explants	MS	1.0	1.0	--	1.0	--	87.5%	(Srichuay *et al*., 2014)
	Lateral Meristems	MS	0.5	--	--	--	0.1	93%	(Srivastava, 2009)
	Inner Teguments	MS	2.0	--	--	0.7	--	88.89%	(Kouassi *et al*., 2013)
	Stalk Explant	MS	2.0	--	--	--	2.0	100%	(Srichuay and Te-chato, 2012)
Bellflower	Petiole Explant	MS	--	0.3	--	--	1.0	100%	(Pirhan *et al*., 2022)
Quinoa	Stem Explants	MS	0.2	--	--	--	0.2	100%	(Telahigue and Toumi, 2017)
	Hypocotyls	MS	2.0	--	--	0.05	--	95%	(Shahin, 2019)
Garlic	Inflorescence Explants	MS	2.0	--	--	1.0	--	96%	(Azopkova, 2023)

	Tip Explant	MS	3.0	0.5	--	--	0.2	91.05%	(Mostafa *et al*., 2020)
	Basal Disc Explant	MS	2.0	--	--	--	2.0	85%	(Haider *et al*., 2015)
	Root Apices	MS	1.0	5.0	--	--	5.0	100%	(Kayalvizhi and Antony, 2011)
Blackberry	Leave Explants	MS	1.5	--	--	--	--	97.06%	(Schmidt-Durán *et al*., 2016)
Watermelon	Cotyledon Segments	MS	--	1.0	--	--	2.0	97.8%	(Zhang *et al*., 2014)
	Coty. Proximal Part	MS	--	--	--	--	3.0	95%	(Cho *et al*., 2014)
Tulip	Bulb Explant	MS	--	--	--	--	0.5	96%	(Maślanka and Bach, 2014)
Tamarind	Cotyledon Explants	MS	--	0.1	--	0.2	0.5	100%	(Mowobi *et al*., 2016)
Winter Cherry	Cotyledon Segments	MS	2.0	--	--	--	0.2	100%	(Rani *et al*., 2003)
	Root Segments	MS	2.0	--	--	--	0.2	100%	(Logesh *et al*., 2011)
	Leaf Explants	MS	3.0	--	--	--	--	94.3%	(Arumugam and Gopinath, 2013)
Broccoli	Stem Explants	MS	--	0.5	--	--	2.0	100%	(Biswas *et al*., 2018)
Cabbage	Cotyledon Explants	MS	1.0	--	--	--	1.0	100%	(Hasan *et al*., 2021)
	Hypocotyl Explants	MS	--	0.5	--	--	4.4	100%	(Gerszberg *et al*., 2015b)
Mustard	Hypocotyl Explants	MS	2.5	--	--	--	--	95.24%	(Lone, 2017)
	Cotyledon Explants	MS	3.0	--	--	--	--	84.60%	(Shyam *et al*., 2021)
Spinach	Hypocotyl Explants	MS	--	7.0	--	--	--	80%	(Arikawa, 1974)
	Root Explants	MS	--	0.9	--	--	2.2	100%	(Nguyen *et al*., 2013)
Guava	Leaf Disc Explants	B5	--	--	--	--	1.5	90%	(Butt *et al*., 2014)
Beetroot	Cotyledon Explants	MS	--	--	--	--	1.0	89%	(Tomita *et al*., 2013)
	Leaf Explants	MS	--	--	--	0.4	--	100%	(De Greef, 1978)
	Root Explants	MS	--	1.0	--	--	0.5	100%	(Wonganu, 2007)
Lettuce	Cotyledon Explants	MS	--	0.5	--	--	1.0	95%	(Mohebodini *et al*., 2011)

	Leaf Explants	MS	--	0.05	--	--	0.4	93%	(Lim *et al*., 2011)
	Hypocotyl Explants	MS	1.0	0.1	--	--	0.5	94%	(Gao *et al*., 2002)
Hazelnut	Cotyledon Explants	MS	2.0	--	--	--	0.2	100%	(Shirazi *et al*., 2020)
Lychee	Anther Explant	MS	3.0	0.5	--	--	0.5	93.06%	(Guo *et al*., 2016)
	Immature Embryo	MS	3.0	--	--	--	--	73.33%	(Qin *et al*., 2024)
	Young Lead Explants	MS	1.5	--	--	--	1.0	83%	(Puchooa, 2004)
Cardamom	Nodal Explants	MS	--	--	--	--	1.5	100%	(Ghazali *et al*., 2021)
	Root Explants	MS	2.0	--	--	--	0.5	75%	(Rao *et al*., 1982)
Ginger	Fresh Bud Explants	MS	2.0	--	--	--	1.0	81%	(Abd El-Hameid *et al*., 2020)
	Shoot Explants	MS	--	--	--	--	1.0	100%	(Miri, 2020)
	Leaf Base Explants	MS	2.0	0.5	--	--	0.5	92%	(Mehaboob *et al*., 2019)
Okra	Hypocotyl Explants	MS	--	2.0	--	--	0.5	95%	(Kabir *et al*., 2008)
	Anther Explants	MS	--	--	2.0	--	2.0	95%	(Ibrahim *et al*., 2023)
	Cotyledonary Nodes	MS	--	0.01	--	--	1.0	100%	(Kabir *et al*., 2016)
Hibiscus	Leaf Explants	MS	0.05	--	--	--	--	80%	(Boodia *et al*., 2009)
Vanilla	Nodal Explants	MS	1.0	--	--	--	1.0	83.3%	(Halim *et al*., 2017)
	Mature Seeds	MS	--	0.5	--	--	1.0	82%	(Divakaran *et al*., 2015)
Pistachio	Stem Explants	DKW	--	0.5	--	2.0	--	96%	(Ghannad *et al*., 2023)
	Inflorescence Explants	WPM	--	1.0	--	--	1.0	100%	(Article *et al*., n.d.)
Chili	Hypocotyl Explants	MS	--	2.5	2.5	--	--	100%	(Yunita *et al*., 2021)
	Cotyledon Explants	MS	--	--	1.0	--	5.0	90%	(Ashrafuzzaman *et al*., 2009)
	Internodal Explants	MS	10.0	--	--	--	2.0	95%	(Khan *et al*., 2011)
Lentil	Shoot Tip Explants	MS	2.0	--	2.0	--	0.8	100%	(Polanco *et al*., 1988)

Periwinkle	Leaf Explants	MS	--	1.5	--	0.5	--	100%	(Akhar and Bagheri, 2013)
	Shoot Tips Explants	MS	--	1.0	--	--	0.5	100%	(Swanberg and Dai, 2008)
Bamboo	Hypocotyl Explants	MS	3.1	--	--	--	2.1	88%	(Li *et al*., 2021)
	Embryo Explants	MS	3.0	--	--	--	0.5	90%	(Devi *et al*., 2012)
	Nodal Explants	MS	0.5	--	--	--	--	100%	(Somashekar *et al*., 2018)
Zucchini	Hypocotyl Explants	MS	2.5	--	--	--	--	90%	(Pal *et al*., 2007)
Bael	Hypocotyl Explants	DCR	2.5	--	--	--	1.0	98.7%	(Dai *et al*., 2023)
	Cotyledon Explants	MS	0.5	--	--	--	0.5	100%	(Hazeena and Sulekha, 2008)
Clitoria	Cotyledon Explants	MS	0.5	--	--	--	--	100%	(Teoh *et al*., 2023)
	Leaf Explants	MS	0.4	--	--	3.8	--	100%	(Mhaskar *et al*., 2010)
Opium Poppy	Cotyledon Explants	MS	1.0	--	--	0.1	--	86.67	(Farjaminezhad *et al*., 2013)
	Seed Explants	MS	1.0	--	--	0.1	--	74.6	(Rostampour *et al*., 2010)
Marijuana	Cotyledon Explants	Daria Ind+	--	0.05	--	1.0	--	100%	(Wielgus *et al*., 2008)
	Leaf Explants	MS	2.0	--	--	--	--	100%	(Ślusarkiewicz-Jarzina *et al*., 2005)
Cashew Nuts	Cotyledon Explants	MS		1.0	--	--	1.00	90%	(Kamshananthi and Seran, 2012)
Lavender	Leaf Explants	MS	2.0		--	--	2.0	100%	(Lavandula, 2014)
	Meristem Explants	MS	--	1.0	--	--	0.5	97.6%	(Yegorova *et al*., 2020)
Rosemary	Leaf Explants	MS	--	0.5	--	--	0.5	88%	(Dong *et al*., 2012)
	Lateral Buds	MS	--	1.0	--	--	0.5	100%	(Al-Saeedi and Al-Rekaby, 2022)
	Leaf Explants	WPM	2.0	--	--	--	0.1	95%	(Aman and Afrasiab, 2014)
Jasmine	Stem Explants	MS	--	1.0	--	--	3.0	89%	(Jasmine and Farzinebrahimi, 2014)
	Leaf	MS	1.5	--	1.5	--	--	91.33%	(Thenmozhi, 2019)
Cumin	Embryo Explants	MS	1.0	--	--	--	--	100%	(Soorni and Kahrizi, 2015)

	Hypocotyl Explants	MS	4.4	--	--	0.2	--	100%	(Mousa *et al*., 2023)
Black Gram	Cotyledonary Nodes	MS	--	--	--	--	10.0	94.67%	(Mony *et al*., 2010)
	Hypocotyl	MS	--	1.3	--	--	--	93.1%	(Lin, 2002)
	Leaf	MS	7.0	--	--	--	--	75%	(Saha *et al*., 2017)
	Shoot Tips	MS	--	1.5	--	--	2.5	86.42%	(Conger and Carabia, 1978)
Pine	Cotyledonary Explants	LP	--	--	0.5	--	5.0	93%	(Schestibratov *et al*., 2003)
	Embryo Explants	MS	3.3	--	--	---	1.1	82.6%	(Diamantoglou *et al*., 1990)
Pearl Millet	Mature Seeds	MS	2.5	--	--	--	--	75%	(Arockiasamy *et al*., 2006)
	Embryo	MS	2.5	--	--	--	0.1	91%	(Oldach *et al*., 2001)
Sweet Clover	Cotyledonary Explants	Fox's Med	1.0	--	--	1.0	--	100%	(Taira *et al*., 1977)
Clove	Leaf	MS	0.5	--	--	--	--	100%	(Rasud *et al*., 2020)
	Cotyledonary Nodes	MS	1.6	--	--	--	--	75%	(Naaz *et al*., 2019)
Cinnamon	Stem Explants	MS	1.5	--	--	1.0	--	89.9%	(Wu Yun, 2010)
Bay Leaf	Lead Bases	MS	1.0	0.8	--	0.1	0.5	62.5%	(Al-Gabbiesh *et al*., 2014)
Coriander	Hypocotyl	MS	1.0	--	--	--	--	96.0%	(Ali *et al*., 2018)
	Embryo	MS	1.0	--	--	--	--	77%	(Kim *et al*., 1996)
Fenugreek	Seed	MS	--	0.5	--	--	2.0	100%	(Agha *et al*., 2022)
Fennel	Hypocotyl Explants	SH	--	0.2	--	--	1.0	92%	(Sharma and Mandal, 2020)
	Shoot	MS	--	1.0	--	0.1	--	100%	(Shima *et al*., 2024)
	Root Explants	MS	--	1.0	--	0.1	--	100%	(Rehab *et al*., 2015)
Ajwain	Hypocotyl	MS	2.0	--	--	0.5	--	100%	(Nomani *et al*., 2021)
	Stem	MS	2.0	--	--	--	0.25	100%	(Fazeli-Nasab, 2018)
Saffron	Lateral Meristems	MS	2.0	--	--	--	0.1	76%	(Vahedi *et al*., 2014)

Age and Size of Explant

Understanding how age and size can affect callus induction can enhance the success rate of tissue culture techniques and optimize the conditions for maize propagation. The chronological age of the explant is a major determinant as it affects different physiological, and biochemical properties in callus production. Normally, younger explants such as immature embryos and young leaves tend to be more prone to callus formation than older tissue (Malini *et al*., 2015; Nadia *et al*., 2021). The young tissues are characterized by higher metabolic activity and cell division. The callus formation is dependent on the pool of proliferating cells and thus immature tissues have a greater number of actively dividing cells. Once the plant

becomes mature, cell differentiation proceeds leading to a decreased portion of meristematic cells that still have the autonomy to keep dividing. Here, older tissues may be less responsive to the hormonal signals required for callus induction. The ability to stimulate plant tissues using plant growth regulators (PGRs), auxins, and cytokinins, can vary with the age of the explants as well. Younger explants are often more sensitive to PGRs which can stimulate callus formation. Conversely, the older tissues may require a higher concentration of PGRs for an equivalent response but might cause tissue damage and reduced viability as well (Abhishek *et al*., 2014). The entire physiology status of the explant at the time of collection is also critical. Younger tissue can better adapt to the *in-vitro* conditions as compared to the aged tissues, probably entering senescence, with a consequent decrease in vigor and ability to callus induction. The size of the explant model is also an important issue that can significantly affect callus formation efficiency. It infiltrates numerous physiological and developmental mechanisms that are directly or indirectly involved in successful callus formation. The small explants usually have a higher surface-to-volume ratio which can promote the uptake of nutrients and hormones from the culture medium. The intensive exposure to growth regulators can further reinforce the induction process, as it makes sure that the signals each explant receives are strong enough for cellular division and differentiation (Ali, *et al*., 2014). Larger explants, however, might not present a large surface area to volume ratio that may limit their sequestration ability for these essential nutrients. Besides the organization of cells and tissues, the internal arrangements of the explants show further effect on callus induction. The smaller explant tissues may more uniformly distribute the cells and therefore even exposure to culture conditions. Alternatively, explants of bigger size may carry inner zones with a reduced presence to the growth regulators that might give place for discrepant callus formation or induction defects in part. The size of the explant can affect its nutritional needs. A larger explant may require higher levels of some nutrients. Large explants need more nutrients to sustain themselves for their growth and development causing the competition for resources in culture medium. This competition can impede the induction process because explants do not obtain enough support for a successful callus formation (Jakubeková *et al*., 2012). The effects of explant size, maturity, and developmental stage on the improvement of callus induction efficiency in maize are important. Ultimately, differential experimental outcomes of callus formation and regenerative potential in different animal models have been empirically shown to depend on the configuration of these components. Studies have shown that small embryo sizes (0.5 to 1 cm) are often the best producers of embryogenic callus in maize under specific conditions (Juárez-González *et al*., 2019). These explants are typically taken from young ears, at the developmental stage. The combination of young and small size contributes to a high surface area-volume ratio that enhances the uptake of PGRs and nutrients by generating vigorous callus growth. The developmental stage of the explant is also crucial. Immature embryos at the right developmental stage offer a singular capacity for callus formation, as may be exemplified. In contrast, explants of tissues developed to a greater extent (e.g., from mature seeds or leaves) may be scarcely responsive under identical hormonal influences (Gorji *et al*., 2011). Thus, it is required that the strict evaluation of size and maturity allows an efficient callus formation and regenerative capacity.

pH of the Medium

The pH of the culture medium directly affects multiple physiological and biochemical processes essential for cellular proliferation, division, and differentiation. Knowledge of the critical influence of pH on callus induction can allow qualitative improvement in tissue culture techniques. The pH affects the availability and solubility of nutrients in a culture medium to a large extent. Nutrients have different solubility profiles at different pH levels (Saldarriaga *et al*., 2014). We know, for example, that micronutrients like iron manganese and Zinc show improved availability at slightly acid pH levels. However, if the pH moves too far from this optimum range it may render these essential nutrients unavailable and hurt callus tissue growth and development. The efficacy of auxins and cytokinin as plant growth regulators (PGRs) also reduces with a shift in pH. Such hormones have the potential to exhibit changes in their ionization state with pH, which would alter their biological activity. For example, auxins are more active when unionized and form predominates only at a slightly acidic pH. The presence of hormones is required to initiate callus formation, thus maintaining an optimum pH will help in keeping these hormones in their active form. pH impacts several metabolic processes inside a cell like respiration, yield, and pH can also be the character of water quality (Pasqua *et al*., 2002; B. N. Sathyanarayana & Blake, 1994). Maintaining the appropriate pH range is essential for cell metabolic functions that are necessary during cellular division and differentiation. Research showed that maize (*Zea mays)* callus induction pH range was from 5.5 to 6.5 (Saldarriaga *et al*., 2014). In this range, nutrients become available to the culture and PGRs can work at a higher potential facilitating callus induction. Although a moderately acidic environment (pH < 5.5) might be favorable, extremely lower pH values will lead to a nutrient imbalance and toxic conditions and eventually depress cellular viability. Increased pH levels (pH > 6.5) may reduce the availability of essential nutrients, especially micronutrients which can limit callus growth *In vitro*. In addition, alkaline pH may affect the ionization of hormones and compromise their action in callus induction efficiency. The pH of the medium affects not only the amount but also the quality of the callus produced. Callus derived at an ideal pH seems to be more

vigor and have better regeneration capacity by further induction of differentiation. Conversely, callus development under non-ideal pH conditions can result in poor growth and differentiation as well as partial loss of viability.

Environmental Factors

In maize, induction of callus is significantly affected by several environmental factors such as variation in temperature, humidity, and the nature of light irradiance. All of these environmental factors have a significant and complex set by which they affect not only the efficiency and effectiveness of the callus that forms but also the rate of callus induction. We believe that a detailed description of exactly how each of these key environmental parameters contributes to maize callus induction will advance our understanding of plant tissue culture. This exploration aims to shed light on the complex interplay between environmental conditions and biological responses in the context of maize callus induction, thereby facilitating improved strategies for tissue culture applications.

Temperature

Temperature is a key environmental factor that could have an important effect on the many physiological and biochemical processes necessary for callus initiation in plants. The metabolic processes of plant cells, which are essential for their proper functioning and growth profoundly influenced by the ambient temperature. While heat can stimulate metabolism and division in maize, it also threatens the proper functioning of cell respiration, photosynthesis, and nutrient uptake — all processes for which temperatures above or below (24°C to 28°C for maize) defined bounds are unequivocally met with decline (Sheridan, 1975; Rafiq *et al*., 2005). On the other hand, a below-optimal temperature leads to slower metabolic processes and as a result, much lower callus induction rates are observed (Duncan and Widholm, 1987). By comparison, temperatures that are too high will place stress on the plant cells which can lead to negative events ranging from tissue necrosis through reduced cell life. The efficiency and mode of action of plant growth regulators (PGRs) are closely linked to the growth temperature conditions, though their function at lower temperatures is poorly understood. Higher temperature increases the solubility and activity of some PGRs, as well as their stimulatory effects on calluses induction. However, it is important to note that if the temperature gets too high there may be a risk of damaging these essential hormones and stopping them from working properly or not allowing callus tissue formation. Extreme temperature variations have a very direct effect on the rates at which callus tissue grows which also affects their development. This can cause a rapid proliferation of callus tissue at optimal temperature levels, while growth rates of the callus are significantly reduced or even prevented altogether if temperatures remain suboptimal (Mostafiz *et al*., 2018). This difference in growth rates may therefore influence the final yield of callus tissue produced, something important to subsequently facilitate regeneration processes.

Humidity

Humidity is an underestimated natural force that cannot be discounted. Plant tissue culture systems for both the induction of callus and the survival of plant tissues are influenced by it. The amount of moisture that is available to the plant tissues in general, depends on how much humidity exists within the culture system. For maize callus induction, the optimum level of humidity is 70-80% (Compton *et al*., 1992). Humidity control is important because it also helps to ensure the presence of turgor pressure in plant cells, providing healthy cell division and growth with good internal support. Alternatively, the explants may desiccate if humidity is too low resulting in a negative impact on callus formation and general tissue viability. The high humidity levels can reduce the transpiration rates as observed in plant tissues. Thus, it helps to reduce the loss of water and therefore helps in maintaining the segregation of cells. Conversely, low humidity can drastically increase transpiration rates and excessive water loss from explants. This can lead to the tissues being dehydrated and wilted, seriously restricting callus initiation and growth. Optimum humidity is a critical defense against diseases caused by pathogens such as bacteria and fungi that can harm plant tissue. High humidity can also create conducive conditions for pathogen development. However, with the right levels of humidity fungal contamination risk can be reduced while ensuring that callus tissues remain healthy, stable, and able to grow.

Light Intensity and Photoperiod

Light is one of the essential environmental factors affecting callus induction by influencing different parameters like light intensity, quality of light, photoperiod, and length of exposure. The formation of callus tissues is promoted by light but general growth and development could be affected by the intensity of light that`s going around. Greater light intensity can also speed up photosynthesis and thus promote the production of energy-rich molecules required for intricate processes of cell growth and metabolism. The key is to remember that too much light will lead to photo-inhibition, which stresses out cells and causes hindrance in callus induction. An optimum level of 80 µM m^{-2} s^{-1} light intensity is reported for maize callus induction (Tiwari *et al*., 2015). Consequently, keeping exactly the right amount of light is essential to orchestrating an ideal environment for callus tissues to grow. Specific ranges of wavelengths that are present hugely affect how plants grow and develop. Specifically, it is discovered that the wavelengths associated

with blue light and red light are most beneficial in supporting plant growth or health. Specific wavelengths are needed to synthesize chlorophyll and other essential pigments, which can promote photosynthetic efficiency and the rapid metabolism rates required for proper callus induction (Ruíz-Rivas *et al*., 2022). Green lights are less effective at promoting plant growth and may not act on the callus formation process. The photoperiod has an important effect on callus induction. Plant tissue types may need exposure to certain light durations for maximum growth and developmental patterns. A nearly equal photoperiod with the surrounding conditions might be supportive for callus induction as it helps synchronize many physiological processes in different explants. It is stated from studies that a photoperiod of 16hr-day and 08hr-night is best suitable for maize callus induction (Astutik *et al*., 2022; Malini *et al*., 2018; Chávez-Hernández *et al*., 2015). In contrast, an extensive period of darkness would inhibit these crucial processes resulting in a decreased capability for callus formation.

FUTURE DIRECTIONS

Although maize tissue culture has made significant progress, there are still several important issues that must be resolved to maximize its effectiveness and usefulness. The genotypic dependence of callus induction and regeneration is one of the main obstacles. Numerous genotypes of maize have poor responsiveness to tissue culture methods, particularly inflexible inbred lines. Finding the genetic and molecular elements that control tissue culture responsiveness should be the main goal of future research. Key genes and processes involved in callus formation, regeneration, and somatic embryogenesis can be identified using techniques like as transcriptomics, QTL mapping, and genome-wide association studies (GWAS). The creation of genotype-independent protocols will guarantee that tissue culture methods may be used more widely for a variety of maize types. The low efficiency of plant regeneration from callus, which continues to be a bottleneck in maize tissue culture, is a further limitation. Because of somaclonal variations and extended subculturing, callus frequently loses its capacity to regenerate over time. Optimized medium compositions, balanced plant growth regulators (PGRs), and cutting-edge methods like liquid culture systems or temporary immersion bioreactors should all be used in research to increase regeneration efficiency. To maintain callus viability and uniformity over the long term, more research is also required to determine the biochemical and molecular causes of callus degeneration and necrosis. More thorough investigation of the effects of environmental factors, including temperature, light intensity, photoperiod, and humidity, in real-time is necessary. Variable outcomes are frequently the consequence of the absence of established procedures to maintain ideal environmental factors. In order to ensure consistency and repeatability in callus induction and regeneration trials, future research should concentrate on automated and precisely controlled methods to manage these parameters. Furthermore, little is known about how environmental stressors like salt and drought affect tissue culture performance. The use of genes linked to stress and research on stress-adaptive systems can aid in the development of maize cultivars that can flourish in challenging environments. Future studies should focus on the use of artificial intelligence and machine learning in precision micropropagation for better *in-vitro* studies. Machine learning models can be trained to optimize the protocols for callus induction with effective PGRs concentrations. Optimization Algorithms (OA), if used in tissue culture, can be helpful in optimizing culture conditions and environmental conditions. Future studies can greatly improve the effectiveness, scalability, and dependability of maize tissue culture by filling up these real-time gaps. In addition to enhancing maize breeding programs, overcoming these obstacles will support agricultural sustainability and global food security.

CONCLUSION

The complex process of callus induction in maize tissue culture is influenced by several biological, chemical, and environmental variables. To ensure strong regeneration potential, explant selection is the most important step. Murashige and Skoog (MS) medium are the most successful across genotypes, and media compositions, which include the proper mix of macronutrients, vitamins, and carbon sources, are crucial. Additionally, the initiation and maintenance of healthy callus development depend on the function of plant growth regulators, particularly auxins like 2,4-D and cytokinins like BAP. The standardization of procedures is further complicated by genotype heterogeneity, polygenic factors, and explant size, underscoring the necessity of genotype-specific optimization. Callus induction is the foundation of plant regeneration, genetic transformation, and somatic embryogenesis, allowing researchers to develop maize varieties that are more durable, high-yielding, and adaptive to changing environmental conditions. In addition to helping farmers, this promotes global food security and sustainable agriculture.

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AUTHOR CONTRIBUTIONS

All authors contributed to the study's conception and design. Data collection and an extensive literature survey were performed by Muhammad Haris, Shahroz Rahman, Ishmal Fatima, Sumbal Rasheed, and Ayesha Mazhar. The first draft of the manuscript was written by Muhammad Haris and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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