# An efficient regeneration with multiple shoots from mature embryo-derived callus of *Zea mays* cvs

**Running Title.** Efficient and enhanced maize regeneration with coconut water

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**Abstract:** *In vitro* propagation and genetic transformation of plants play a vital role in both basic and applied research. *Zea mays* are one of the most important cereal crops reluctant to *in vitro* regeneration. In maize genetic modification, the most important is an effective tissue culture protocol capable of efficient and reliable callus induction and regeneration. Callus induction and regeneration ability of maize cultivars: Neelam, Dehkan, and Pak Afghoi mature embryos were monitored. Callus induction and regeneration were genotypes, medium and auxin, cytokinin, and coconut water-dependent. The highest callus induction: was achieved, with 2, 4-D (3mgL$^{-1}$), and BAP (2mgL$^{-1}$). 2,4-D (0.5mgL$^{-1}$) in combination with BAP (2.0 mgL$^{-1}$) presents maximum regeneration efficiency was 48% for Neelam, 53% for Dehkan and 42% for Pak Afghoi. Regeneration was enhanced with 20% coconut water in combination with 2, 4-D (0.5 mgL$^{-1}$), and BAP (2.0 mgL$^{-1}$). It was 54% for Neelam, 60% for Dehkan and 43% for Pak Afghoi. Before, the high embryogenic callus production and regeneration are not reported with coconut water.

**Index Term:** Maize, Regeneration, 2, 4-D, coconut water, BAP, Callus, Embryo

**Abbreviations**: Indole acetic acid (IAA); 2, 4-Dichlorophenoxyacetic acid (2, 4-D); 6-Benzyl aminopurine (BAP); Coconut water (CW); Murashige and Skoog (MS).

## 1. Introduction

Maize is an important cereal crop becoming next the main staple crop. The maize demand is increasing across the world, especially in Asia [1]. Millions of people living in tropical and subtropical regions are directly dependent on corn. Due to its C4 activity and more photosynthesis than other crops, it produces more yield and biomass for industries and becomes vital for human and animal food [2], [3]. Approximately 50% of the human population utilizes corn as a staple food in many developed and developing countries [2]. Maize is affected by many biotic and abiotic factors [4], [5]. A time constrain is connected to conventional breeding: needs alternatives to plant resistance against environmental stresses. Gene transformation is an alternative, that requires tissue culture optimization [6]. The transgenic varieties against biotic and abiotic stresses enhance productivity and nutritional quality. Genome variations, genotype, explant source, culture media, and environmental conditions are parameters for tissue culture optimization [7]. In this regard, monocots are diehard: and due to deficient reproducible and efficient regeneration, maize is most difficult compared to others. Maize regeneration with anther, meristem, stem tips, and mature and immature embryos are reported [8; 9; 10]. The corn embryo is more reported due to easy inoculation, more callus production, and regeneration of secondary culture; but still in infancy [11], [12]. Genome and exogenous hormones like abscisic acid, indole acetic acid (IAA), 2, 4-Dichlorophenoxyacetic acid (2, 4-D), and gibberellic acid are reported [13], [14].

Coconut water (CW) had the auxin and cytokinin-type activity (Zeatin riboside, isopentenyl adenine, IAA, and kinetin), along with a source of sugars, vitamins, minerals, and amino acids; which minimizes the cost of plant propagation [15]; [16], [17]. Embryogenic callus and micropropagation with CW are reported in olives, sweet potato, *Celosia,* and *Corylus avellana* L [18], [19], [20], [21]. CW for enhanced corn regeneration using maize mature embryos is novel, with the least number of growth regulators. The effect of different growth regulators for callus induction and plant regeneration ability

of three maize genotypes (Neelam, Dehkan, Pak Afghoi) was enhanced and optimized. The optimized protocol will be helpful for maize transformation studies.

## 2. Research Elaborations

### 2.1. Plant material and sterilization

Mature *Z. mays* var (Neelam, Dehkan, and Pak Afghoi) seeds were obtained from Horticultural Research Institute (HRI); National Agriculture Research Centre (NARC) Islamabad. 2g seeds of all varieties were surface sterilized with a few drops of Tween-20 followed by washing in 0.8% v/v sodium hypochlorite (Clorox), for 20 min. It was followed by seeds washing with DD water for 15 min thrice. Seeds embryos were placed on the appropriate media.

### 2.2. Callus induction and regeneration

Murashige and Skoog (MS) medium [22] was prepared along with vitamins including thiamine, myoinositol, nicotinic acid, pyridoxine, and glycine. MS media and vitamins with (1, 2, 3, and 4 mg $L^{-1}$) 2, 4-D, and BAP (0.5, 1, 2, and 2.5 mg $L^{-1}$), were used for callus induction, with 30 g $L^{-1}$ sucrose as a carbon source; and 6 g $L^{-1}$ agar as the gelling agent. The pH was adjusted to 5.8. Inoculated embryos were placed in the growth chamber for 2D without light, followed by a 2W light period under standard conditions (300 µmol $m^{-2}$ $s^{-1}$, 16 h light/8 h dark at 25°C and 70% relative humidity). Then calli (0.5 cm size) was transferred to a maintenance MS medium with 2, 4-D (2 mg$L^{-1}$). After two weeks; the maintained proliferated calli were transferred to a regeneration medium containing 2, 4-D, (0.5 mg $L^{-1}$), and BAP (0.5, 1, 2, and 2.5 mg $L^{-1}$). The best combination obtained was used with CW (10, 15, 20, and 25%). Cultures were transferred to the growth room under standard conditions. For callus induction and regeneration, four replications (100 explants/Cali/replication) were used: in each treatment. Means and STDEV are used; for error bars in MS Excel. One-way analysis of variance; was used to find the significant difference between the different concentrations of growth regulators responses in different maize varieties. Different alphabets reflect the significant difference among varieties within the same media composition.

## 3. Result and Discussion

Tissue culture techniques are employed: to improve plant growth by medicinal and ornamental plants, biological activities, transformation, and secondary metabolites production [13]. Maize is one of the world's three most important staple crops with wheat and rice and is gaining more importance on a day-to-day basis worldwide basis. Its importance will be more prominent in the next decade, especially for health-conscious people in basic; or processed forms [23]. Biotechnology's importance is for maize genetic improvement and augmented production with its expanding demands. One of the main aims of maize tissue culture is to use maize germplasm for transforming immature/mature embryos with *Agrobacterium tumefaciens*, using standard binary vectors/RNAi constructs [9], [10], [24], [25], [26]. The first maize regeneration from immature embryos leading to callus induction was reported in 1975, followed by embryo-derived maize tissue cultures from scutellum cells via somatic embryogenesis [11], [12], [27].

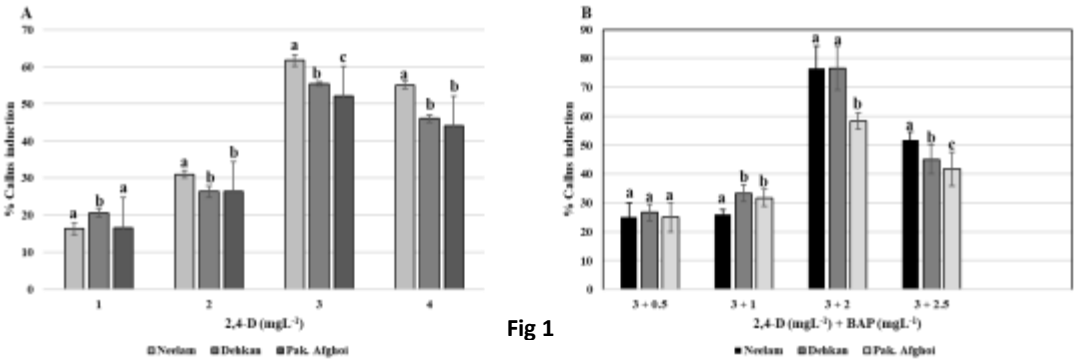


Fig 1

**Fig 1. (1A) Effect of 2, 4-D on the callus induction of *Z. mays* cvs (Neelam, Dehkan, Pak Afghoi) (1B) Effect of 2, 4-D and BAP on the callus induction of *Z. mays* cvs (Neelam, Dehkan, Pak Afghoi).**

In this study, callus induction; and regeneration of maize: a reluctant crop by immature embryos: were studied. Embryogenic callus induction in maize is crucial, as regeneration from non-embryogenic callus is negligible [23]. The molecular mechanism involved in the maize cultivars' differential response for callus induction and regeneration: shows increasing transcripts related to signal transmission, transduction, iron and heme-binding, membrane formation, defense, and energy transduction, via GA signaling [28], [29]. Du et al. [29] reported ZMBBM; importance in the signal transduction pathway, leading to cell differentiation and somatic embryo formation; confirmed by the transformation of an immature embryo. Although immature embryo-derived callus is frequently reported: constraint is the unavailability of explants round the year, pollination seasonal variation, and greenhouse facility requirements [14]. Maize cultivars *in vitro* cultures differences are genotypic dependent; so maize varieties (Neelam, Dehkan, Pak Afghoi) callus induction and regeneration were optimized using a combination of 2, 4-D, and BAP. It was followed: by callus regeneration by combinations of 2, 4-D, BAP alone, or combination to CW. Factors affecting callus regeneration include genotype, medium, type, and level of auxin. 2, 4-D higher concentration has a significant role in callus induction; hinders regeneration.

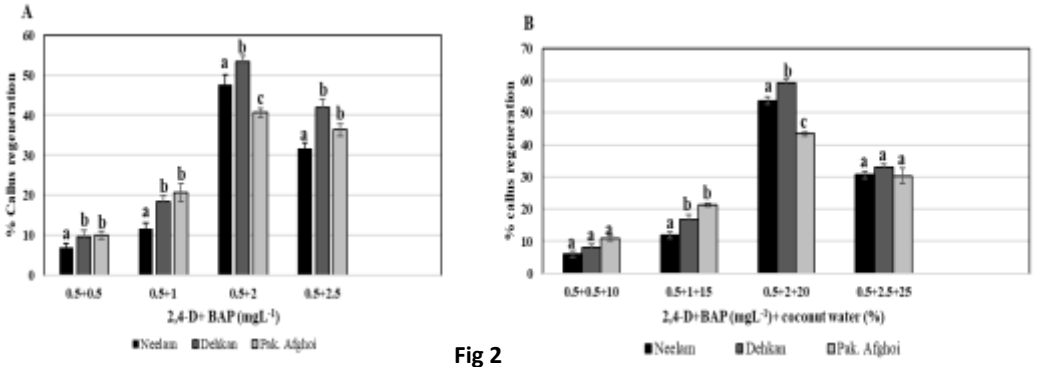


Fig 2

**Fig 2. (2A) Effect of 2, 4-D, and BAP on the callus regeneration of *Z. mays* cvs (Neelam, Dehkan, Pak Afghoi). (2B). Effect of 2, 4-D, BAP, and coconut water on the callus regeneration of *Z. mays* cvs (Neelam, Dehkan, and Pak Afghoi).**

## 3.1. Influence of plant growth hormones on callus induction and regeneration

*Z. mays* cvs Neelam, Dehkan, Pak Afghoi mature embryos had differential responses on a combination of MS media. Mature embryos forming abnormal callus were removed manually and sub-cultured for further proliferation. 2, 4-D (3mg

$L^{-1}$) gave the highest callus induction percentage: 62% for Neelam, 55% for Dehkan, and 52% for Pak Afghoi; among all combinations used (Fig 1A, 3A). It was further improved, with BAP (0.5, 1, 2, and 2.5 $mgL^{-1}$): and maximum callus induction was observed with 3$mgL^{-1}$ 2, 4-D and 2.0 $mgL^{-1}$ BAP was: Neelam (77%), Dehkan (76%), and Pak Afghoi (58%) (Fig 1B, 3). 90% and 52.5% callus induction: were reported with 2, 4-D (3 mgL-1) alone or in combination with kinetin [30]. Du *et al*. [29] reported immature embryos (1.0-1.2 mm) on an N6 medium with 1.5 $mgL^{-1}$ 2, 4-D for transcriptomic analysis for understanding the mechanism involved in the callus induction for reluctant maize cultivars. 2, 4 D higher concentration was essential for callus induction from cereal embryos.

**Fig 3**

**Fig 3. Callus induction of *Z. mays* cvs (Neelam, Dehkan, Pak Afghoi) on 2, 4-D (0.5$mgL^{-1}$) in combination with BAP (2.0 $mgL^{-1}$) 3 weeks' post-inoculation (3A, 3F) Neelam (3B, 3C) Dehkan (3C, 3D, 3E) Pak Afghoi.**

Dehkan gave the highest regeneration against all other cultivars for all growth regulator combinations. 0.5$mgL^{-1}$ 2, 4-D and 2$mgL^{-1}$ BAP: represent the highest regeneration: 48% for Neelam, 53% for Dehkan and 42% for Pak Afghoi (Fig 2A). Calli regeneration was further improved with CW (10-25%) in addition to 0.5$mgL^{-1}$ 2, 4-D and 2$mgL^{-1}$ BAP. The highest regeneration was observed: 2, 4-D (0.5 mg $L^{-1}$) and BAP (2 mg $L^{-1}$) with 20% CW: Neelam (54%), Dehkan (60%), and Pak Afghoi (43%) (Fig 2B, 4, 5). LS medium for maximum maize callus induction with 1.5 mg $L^{-1}$ 2, 4-D is reported. BAP and Kinetin (0.5 mg $L^{-1}$, each): are reported for regeneration [24]. 16-21% regeneration was found: on MS media having 4 mg $L^{-1}$ BAP and 2 mg $L^{-1}$ Kinetin [30].

Regeneration and callus induction in maize is highly genotypic dependent, and the genotype determines the callus induction percentage. Various reports tell us widely about the combinations of growth regulators with basal medium [11], [12]. MS media with 2 mg $L^{-1}$ BAP, 1 mg $L^{-1}$ Kinetin and 0.5 mg $L^{-1}$ naphthalene acetic acid promoted the highest frequency of shoot induction in maize [31]. Hong *et al*. [32] reported immature embryos on MS media proline, dicamba with 2, 4-D.

**Fig 4**

**Fig 4. Effect of 2, 4-D (0.5mgL$^{-1}$) in combination with BAP (2.0 mgL$^{-1}$) on the callus regeneration of *Z. mays* cvs. (4A, 4B, 4C). Effect of 2, 4-D, BAP, and coconut water on the callus regeneration of *Z. mays* cvs (Neelam, Dehkan, and Pak Afghoi) 2 weeks and (4D, 4E, 4F) 3 Week-Post-Inoculation.**

(0.5 mgL$^{-1}$), and AgNO3 for the highest callus induction. While MS medium supplemented: with zeatin (5 mgL$^{-1}$) shows the highest regeneration. Jiao et al. [33] reported $N_6$ medium supplemented with 0.25 mg L$^{-1}$ 2, 4-D, proline (0.8 mg m L$^{-1}$), and folate 0.5 mg mL$^{-1}$ for maize callus induction (84%.) IBA 0.8 mg L$^{-1}$ and 6-BA 1.5 mg L$^{-1}$ were added: to the $N_6$ medium in regeneration. N6 supplemented with 2, 4-D (2 mg L$^{-1}$): reported for embryogenic callus induction. And regeneration percentage of 12.2% was reported with MS medium, 60% sucrose and IAA 0.5 mg L$^{-1}$ + BAP 1.0 mg L$^{-1}$ [34]. Similarly, we have obtained higher callus induction with higher 2, 4-D, and BAP concentration (3 mgL$^{-1}$). Regeneration maximum frequency was obtained: with lower auxin (0.5 mg L$^{-1}$) and relatively higher BAP concentration (2.0 mg L$^{-1}$) with 20% CW (Fig 2, 5). CW is one of a complex substance: a mixture of auxins and cytokines [15], [16], [17]. So, it can replace the expensive growth regulators; for the recalcitrant crop regeneration. CW in maize regeneration is reported: in the late '90s, but chimera callus growth is reported rather than the compact callus [35].

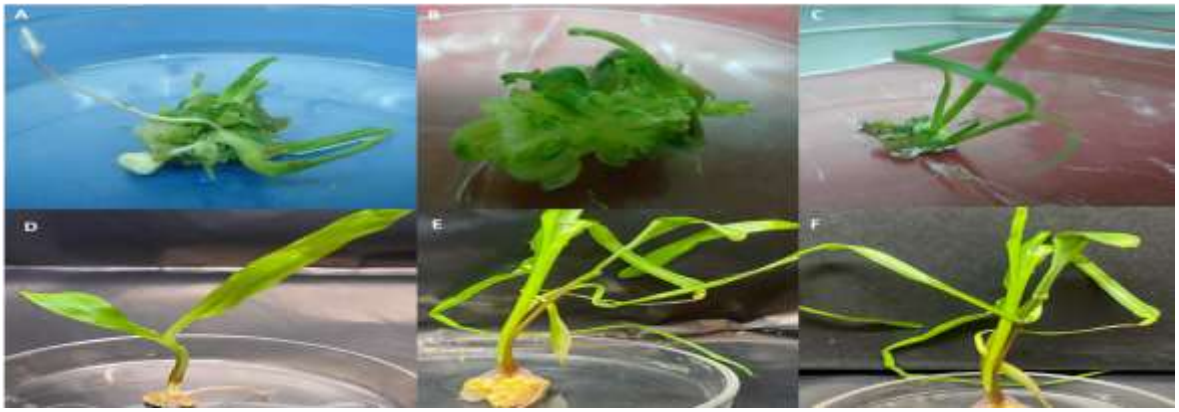

**Fig 5**

**Fig 5. Regeneration of *Zea mays* cultivar (Neelam, Dehkan, Pak Afghoi) on 2, 4-D, BAP, and coconut water (5A) Neelam (5B) Pak Afghoi (5C) Dehkan, followed by shifting to fresh medium for regeneration on (5D) Neelam (5E) Pak Afghoi (5F) Dehkan.**

## 4. Conclusion

At the stage of embryogenic callus induction, induction rate and regeneration ability are controlled by genetic factors of embryogenic callus. Different genotypes e.g. Neelam, Dehkan, and Pak Afghoi showed varying results, while embryo induction of Dehkan was the most successful for regeneration (53-60%). The highest callus induction: was achieved with

2, 4-D (3mgL$^{-1}$), and BAP (2mgL$^{-1}$). Regeneration was enhanced with 10% CW in combination to 2, 4-D (0.5mgL$^{-1}$), and BAP (2.0 mgL$^{-1}$).

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## Conflicts of Interest
The authors declare no conflicts of interest.

## Ethical Statement
This article does not contain any studies with human participants or animals performed by the authors.

## Consent for publication
The corresponding author had taken consent from all co-authors to the submission, and publication of their data in the Journal of Xi'an Shiyou University, Natural Science Edition.

## Author Contribution
Atif Mehmood (Experiment execution, Formal analysis, First draft preparation); Dr. Amber Afroz (Supervision, Experimental Design, Concept, Funding source); Dr. Umer Rashid (Co-supervision, Concept, Paper Final editing).

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