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RESEARCH ARTICLE

# Direct and indirect organogenesis in soybean for efficient shoot induction through balanced levels of auxins and cytokinins

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## Abstract

Soybean (*Glycine max* L.) is an essential oilseed crop widely recognised for its high protein and oil content. Soybean (Co1) is a high-yielding variety and well-suited for cultivation in Tamil Nadu and nearby regions. Direct and indirect organogenesis of soybean using different growth regulators is helpful for regeneration, micropropagation and genetic transformation. We evaluated the impact of various plant growth regulators (BAP, KIN, ZT and TDZ) for direct organogenesis using split seeds and cotyledonary nodes of soybean (Co1). Among the different combinations, B5 medium with BAP 1.0 mg/L (for multiple shoots) and KIN 1.0 mg/L (for shoot length) revealed the better outcomes for the direct shoot rejuvenation in the split-seeds. The highest frequency of multiple shoots from the cotyledonary node was induced in MS medium containing TDZ at 0.5 mg/L. Different levels of 2,4-D have been assessed for callus induction from various explants and a higher percentage of callus was developed in hypocotyls. MS media containing 2,4-D (2.0 mg/L) and KIN (0.2 mg/L) leads to better callus development and the friable callus was further used for shoot induction using BAP, TDZ and ZT. After 2 - 3 weeks, initiation of shoot formation from embryonic callus was observed in MS medium supplemented with 1.0 mg/L ZT and 0.5 mg/L IAA. Shoot induction from split seeds showed better response than the cotyledonary node and callus. These findings are expected to be useful for the regeneration of soybean (Co1) to develop transgenic lines for enrichment of nutritional value.

**Keywords:** callus; cotyledonary node; shoot induction; soybean; split-seed

## Introduction

Soybean (*Glycine max* (L.) Merr.) belongs to the Fabaceae family and is well known for its protein and oil content. The annual production of soybeans (395 MMT), Soybean oil (63 MMT) and soybean meal (260 MMT) was recorded in the year 2023-24. Soybean contributes to more than 60 % of global oilseed production and, therefore, genetic improvement of soybean varieties is crucial for sustainable agriculture (1). Organogenesis is a process of organ formation from plant tissues or cells under *in -vitro* conditions. Of the two pathways of regeneration, direct organogenesis involves organs developing directly from explants without involvement of callus and thus provides genetic stability and higher efficiency of regeneration. In contrast, indirect organogenesis involves the formation of a callus that can be differentiated into shoots and roots, allowing for genetic manipulation (2, 3). The success of genetic transformation is dependent on efficient gene transfer and plant regeneration protocols (4).

Split-seed explants were obtained from overnight imbibed seeds, which have a partial embryonic axis attached to them. Regeneration involving this explant is simple and does not require a long-term tissue culture procedure. It is suitable for basic research and development of transgenic soybean (5). A high efficiency of shoot regeneration was achieved through direct organogenesis from cotyledonary node and hypocotyl segments as a suitable explant choice for plant regeneration in soybean (6). Cotyledonary nodal explants were obtained from aseptically grown seedlings by removing cotyledons, epicotyl and hypocotyl (7). Organogenesis causes shoot buds to emerge from cortical cells, resulting in the rapid development of plantlets (8). Callus is an ideal choice for the various research experiments, mass propagation, efficient transformation, selection and regeneration of the cells (9). Callus is more desirable for plant genetic transformation than direct regeneration, as it allows for better selection of homogeneous transgenic plants (10).

The most common phytohormones are cytokinins and auxins, which either directly or indirectly promote rejuvenation of shoots (11). Although studies have explored shoot induction using split seeds, callus and cotyledonary nodes, there is a lack of comprehensive research comparing the efficiency of these explants under various conditions. Identifying the most suitable explant for consistent and high-frequency shoot induction remains a critical area of investigation. Therefore, the study focuses on the evaluation of various plant growth regulators on direct and indirect organogenesis in soybean cv. Co1. The variety

Co1 is developed by the Tamil Nadu Agricultural University, which is a re-selection from a Thailand variety that exhibits the duration of cultivation from 90-100 days. Hence, the current study aims to provide insights into optimization of protocol for efficient regeneration using different types of explants such as split seeds, cotyledonary nodes and callus explants.

## Materials and Methods

### Split seed regeneration

Soybean seeds (Co1) were collected from the University of Agriculture, Dharwad, Karnataka, India. The seeds were immersed in water for 16 hr in the dark at 25 °C. After soaking, the seeds were sterilized with 0.12 % $HgCl_2$ for 60 sec, followed by 0.1 % NaOCl for 60 sec and then washed with sterile water. Split-seed explants were prepared by excising the seed coat and longitudinally dividing the seeds into two halves, one containing the embryo and the other containing only the cotyledon (5). The cotyledon, which includes the embryonic axis (consisting of radicle and a part of hypocotyl), was partially trimmed by removing 1/3 of the embryonic axis (Fig. 1a). The sectioned split seeds were blot-dried and inoculated on B5 Gamborg medium supplemented with 3 % sucrose, 0.8 % agar and varying combinations of cytokinins such as kinetin (KIN), 6-benzylaminopurine (BAP), zeatin (ZT) and thidiazuron (TDZ) in combination with auxin, namely, naphthaleneacetic acid (NAA) (Table 1) (12). The pH of the medium was adjusted to 5.7 before autoclaving. All chemicals and growth promoters used in the medium were procured from HiMedia (India). The cultures were kept in the dark at 25 °C for two days. Following dark incubation, the split seeds were cultured under a 16-hour photoperiod followed by 8 hours of dark incubation at 25 °C. Data on the number of shoots per explant and shoot length (cm) were recorded for all treatments and subjected to analysis.

### Cotyledonary node regeneration

Cotyledonary nodes from germinated seedlings (Fig. 1c) were used as explants for direct shoot induction. The explants were sterilized and then cultured on MS medium supplemented with cytokinins and auxins (Table 1). The number of shoots per explant and shoot length (in cm) were recorded in all the treatments for analysis.

### Callus and shoot induction

Seeds soaked in double-distilled water and kept at room temperature in the dark for 3 hours were surface sterilized and placed on clean filter paper for blot drying. Then the blot dried seeds were inoculated in half-strength MS medium (13). For germination, the cultures were initially incubated in complete darkness for 48 hours, then transferred to a photoperiod (16:8 hours) at 25 °C. From the germinated seedlings, explants were dissected after 8 to 10 days. For callus induction, various explants such as leaf, hypocotyl, cotyledonary node and cotyledon were used (Fig. 1b-e). The explants were surface sterilized following the same procedure as for split seeds and inoculated on MS medium containing varying concentrations of 2,4-D (2,4-dichlorophenoxyacetic acid) and KIN (2,4-D 1 mg/L + KIN 0.1 mg/L; 2,4-D 2 mg/L + KIN 0.2 mg/L; 2,4-D 3 mg/L + KIN 0.3 mg/L), along with 3 % sucrose and 0.8 % agar. The pH of the medium was adjusted to 5.7. The cultures were incubated under a 16-hour light and 8-hour dark cycle at 25 °C. Observations were made on callus induction percentage, callus texture, colour and weight.

Well-developed friable callus was utilized for indirect shoot induction. The callus was subcultured on MS medium supplemented with varying concentrations of cytokinins, 0.5 mg/L indole-3-acetic acid (IAA), 30 g/L sucrose and 8 g/L agar, with the medium pH adjusted to 5.7 (Table 1). The cultures were incubated in darkness for two weeks before being transferred to a 16-hour photoperiod and 8-hour dark cycle at 25 °C.

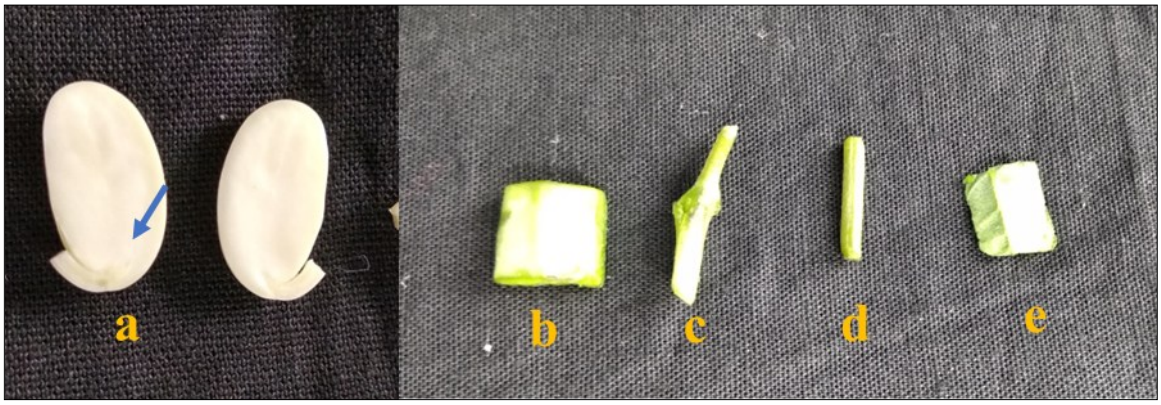



**Fig. 1.** Explants used for shoot induction and callus induction: a) Split seed with embryo; b) Cotyledon; c) Cotyledonary node; d) Hypocotyl; e) Leaf.

**Table 1.** Various concentrations of cytokinins and auxins used for shoot induction in explants, including split seeds, cotyledonary nodes and callus

| Split seeds treatment (mg/L) | | Cotyledonary node treatment (mg/L) | | Callus treatment (mg/L) | |
|---|---|---|---|---|---|
| $S_1$ | BAP 0.5 + NAA 0.25 | $C_1$ | BAP 2 +NAA 0.5 | $T_1$ | BAP 1.0 + IAA 0.5 |
| $S_2$ | KIN 0.5 + NAA 0.25 | | | $T_2$ | KIN 1.0 + IAA 0.5 |
| $S_3$ | TDZ 0.5 + NAA 0.25 | $C_2$ | KIN 2 +NAA 0.5 | $T_3$ | TDZ 1.0 + IAA 0.5 |
| $S_4$ | ZT 0.5 + NAA 0.25 | | | $T_4$ | ZT 1.0 + IAA 0.5 |
| $S_5$ | BAP 1.0 + NAA 0.5 | $C_3$ | TDZ 0.5 +NAA 0.5 | $T_5$ | BAP 2.0 + IAA 0.5 |
| $S_6$ | KIN 1.0 + NAA 0.5 | | | $T_6$ | KIN 2.0 + IAA 0.5 |
| $S_7$ | TDZ 1.0 + NAA 0.5 | $C_4$ | ZT 0.5 +NAA 0.5 | $T_7$ | TDZ 2.0 + IAA 0.5 |
| $S_8$ | ZT 1.0 + NAA 0.5 | | | $T_8$ | ZT 2.0 + IAA 0.5 |

### Statistical analysis

Completely Randomized Design (CRD) and ANOVA were carried out for the significance. Mean values were predicted using Duncan's Multiple Range Test (DMRT). For statistical analysis, SPSS (Statistical Package for the Social Sciences) software version 17.0 and the R program version 4.4.2 were utilized.

## Results and Discussion

### Split seed regeneration by direct organogenesis

Of the different cytokinins, BAP (1.0 mg/L) was the most potent cytokinin for promoting multiple shoots (3.00 shoots/explant) in the split seeds (Fig. 2). BAP showed a superior effect on shoot induction in half seeds after 45 days of culture in the soybean crop (14). The most effective shoot induction was achieved directly in apical or axillary buds using a BAP and NAA combination (15, 16). BAP enhanced the development of multiple shoot buds from a single explant. This can be achieved by removing the axillary bud or injuring the emerging apical bud, leading to the break of apical dominance, a phenomenon where it arrests the main shoot by promoting lateral buds. By promoting the growth of lateral buds, BAP allows the formation of multiple shoots, which is highly desirable in plant tissue culture for *in-vitro* clonal propagation. KIN showed moderate effectiveness, particularly at 1.0 mg/L. TDZ and ZT had relatively low impact on shoot formation, with minimal differences between the two concentrations tested. The control treatment had the lowest number of shoots, indicating the necessity of cytokinin for enhancing shoot development. KIN at 1.0 mg/L resulted in the longest shoots (7.70 cm), followed by ZT with 7.38 cm. The shoot length and number of shoots per explant were increased with an increase in concentration of KIN (Fig. 2) (17). TDZ consistently produced the shortest shoots across both concentrations (Fig. 3). TDZ in the medium was reported to cause abnormal growth and development, such as hyperhydricity, short and compact shoots and hamper shoot elongation and rooting (18, 19). This result reveals the contrasting effects against cytokinin, where BAP enhanced multiple shoot proliferation while KIN and ZT are more effective in promoting shoot elongation (Fig. 3).

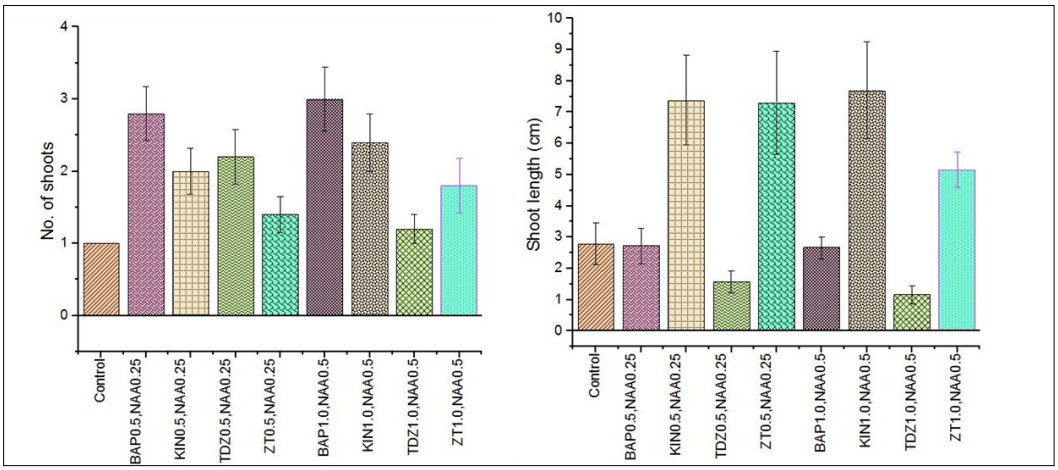



**Fig. 2.** Effect of different cytokinins with NAA in the split seed shoot induction.

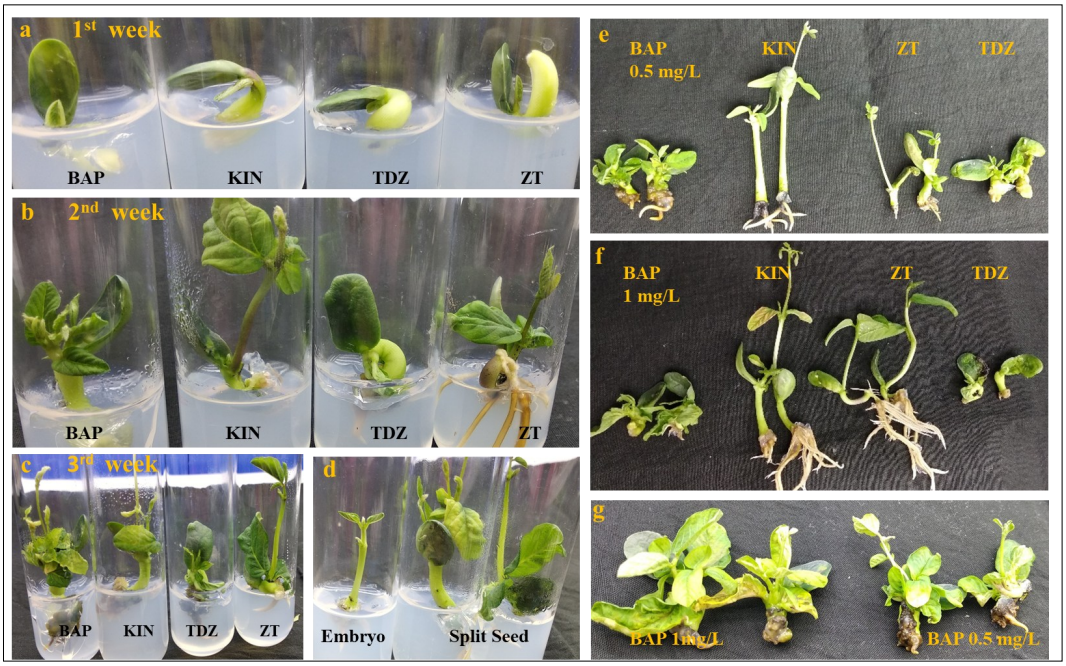



**Fig. 3.** Effect of different concentration of cytokinin with NAA in the split seed direct organogenesis a) one week old split seed in the B5 medium with 1 mg/L of different cytokinin with 0.5 mg/L NAA; b) two weeks old split seeds; c) three weeks old split seeds; d) *in-vitro* germinated embryo and split seed of soybean (Co1); e) proliferated shoots from split seed in the B5 medium with 0.5 mg/L of different cytokinin with 0.25 mg/L NAA; f) 1.0 mg/L of different cytokinin with 0.5 mg/L NAA; g) induction of multiple shoots in BAP 1 mg/L with NAA 0.5 mg/L and BAP 0.5 mg/L with NAA 0.25 mg/L.

### Cotyledonary node regeneration by direct organogenesis

Cotyledonary nodes of 1 to 1.5 cm were responded well to direct organogenesis. Of the different cytokinin with NAA, TDZ (0.5 mg/L each) + NAA (0.5 mg/L) has responded well with higher number of shoots (2.25/explant) and with the longest shoot length (9.38 cm) (Fig. 4). Cotyledonary nodes cultured on the medium supplemented with 0.1 mg/L TDZ, induced more number of shoots (20). Both BAP (2 mg/L) and ZT (0.5 mg/L) in combination with NAA (0.5 mg/L) increased the number of shoots compared to the control. KIN (2 mg/L) with NAA (0.5 mg/L) showed no improvement in number of shoots/explants but with increased shoot length (Fig. 5). Results were in line with the findings of numerous studies, which had shown that MS media when added with 2 to 3 mg/L of BAP, promotes multiple shoot formation from cotyledonary nodes (21, 22).

### Indirect organogenesis by callus induction

For the indirect organogenesis, callus was induced using varying proportions of 2,4-D with KIN in different types of explants. The hypocotyl explant exhibited the highest potential for callus formation across the combinations (Fig. 6). The cotyledon and cotyledonary nodal explants also demonstrated good callus induction with a steady increase in callus weight as the concentration of 2,4-D and KIN increased. Leaf explants produced the lowest callus weight but showed some improvement with higher concentrations of 2,4-D and KIN. Previous study suggested that 2,4-D can be used as an auxin source to induce callus in legumes at 1-2 mg/L, being the most efficient concentration (23, 24). 2,4-D promotes rapid and uncontrolled cell division, leading to the formation of undifferentiated cells. The resulting callus were creamy white (Fig. 6). These calli lack the specialized structures required for chlorophyll production, contributing to the colourless appearance of the callus. 2,4-D is an effective auxin that promotes callus development in various explants. The combination of 2,4-D with BAP and KIN played a crucial role in promoting callus proliferation from anthers of the two different soybean cultivars (25). Among the tested auxins with cytokinin, the combination of 2,4-D 2 mg/L with kinetin 0.2 mg/L produced superior results (Fig. 7). Various ratios of 2,4-D with kinetin have resulted in higher proliferation of callus (26).

The actively growing, friable callus was subcultured into shoot induction media with varying concentrations of cytokinins and auxins. All cultures were incubated in darkness for two weeks. Cultures shifted to photoperiod condition (16:8 hrs) after 2-3 weeks had responded for shoot initiation (Table 2). When the callus was cultured in shoot-inducing medium (SIM) containing elevated cytokinin with minimal auxin levels under light conditions, greenish callus development was observed, which is expected to produce adventitious shoots. Conversion of greenish proliferated callus tissue into shoot-forming cells is crucial because adventitious shoots arise from these progenitor cells (27).

Among the different treatments, most of the callus were found to induce roots in the combinations and only ZT with IAA responded to shoot induction (Table 2). The optimal regeneration rate was achieved using MS-B5 medium nourished with 0.5 mg/L zeatin and 0.5 mg/L IAA. However, the maximum number of shoots from a single explant was examined when 1 mg/L zeatin was used alone (28). The presence of exogenous auxin notably elevates the impact of ZT on both callus induction and the initiation of new shoots (29). The combination of 2.0 mg/L ZT and 0.1 mg/L IAA was reported to induce maximum shoots from the cotyledon in the tomato cultivars (30).

After a fortnight of incubation in shooting media, fluffy white powdery structures were observed on the surface of the callus (Table 2). To induce callus, explants were inoculated in auxin-dominant and reduced cytokinin callus-inducing medium (CIM) and incubated in darkness, which is expected to promote root induction as reported in the earlier studies (31, 32). The freshly developed callus resembled a group of root primordia-like cells as similar to previous reports (32, 33).

## Conclusion

A protocol for *in vitro* regeneration with higher efficiency involving minimum time requirement is necessary for genetic improvement and functional genomics studies. Soybean is one of the crops where organogenesis is one of the challenging issues. Among various methods employed for shoot induction using different plant growth regulators, the split-seed explant method is found to be a more efficient and simpler process. This method is more time-saving as compared to indirect organogenesis method through callus formation. By adopting the split-seed method, it is feasible to achieve high-throughput production of transgenic plants through an efficient genetic transformation technique to complement traditional breeding programs of soybean. Split-seed explant method offers a faster approach to regenerate plants, making it a preferable choice for producing a large number of genetically modified plants within a shorter time.

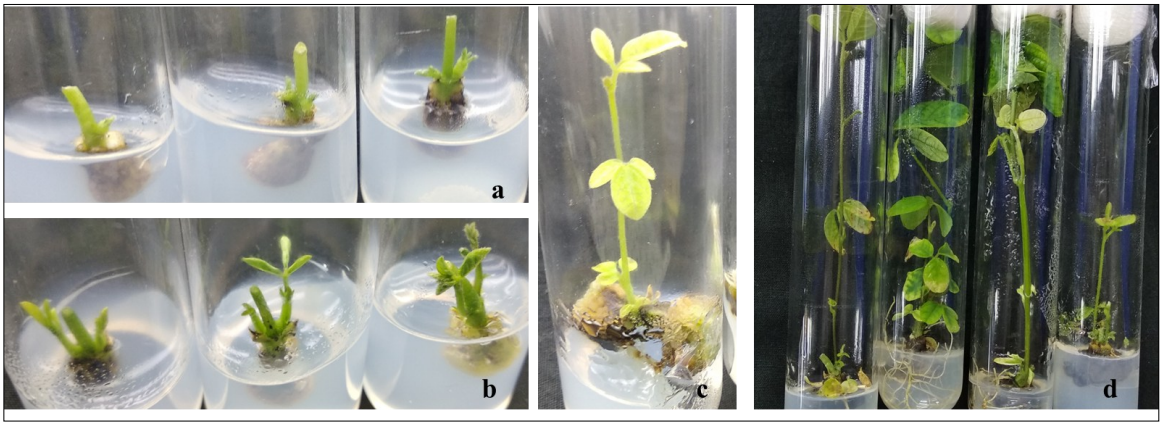



**Fig. 4.** Effect of different concentrations of cytokinin with NAA in the cotyledonary node direct organogenesis a) One-week-old cotyledonary node in the MS medium with TDZ 0.5 mg/L+ 0.5 mg/L NAA; b) Two weeks old; c) Three weeks old; d) Fully elongated shoots.

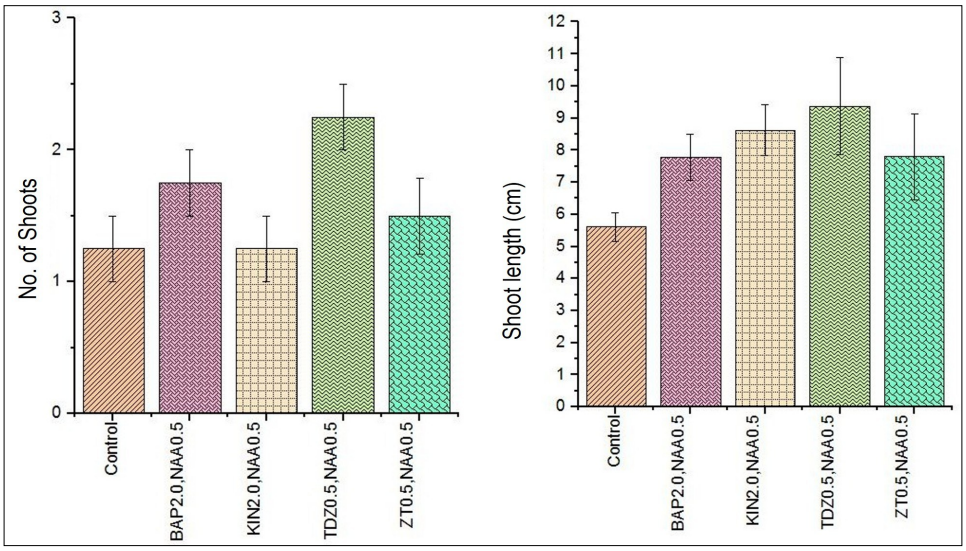



**Fig. 5.** Effect of different cytokinins with NAA in the cotyledonary node shoot induction.

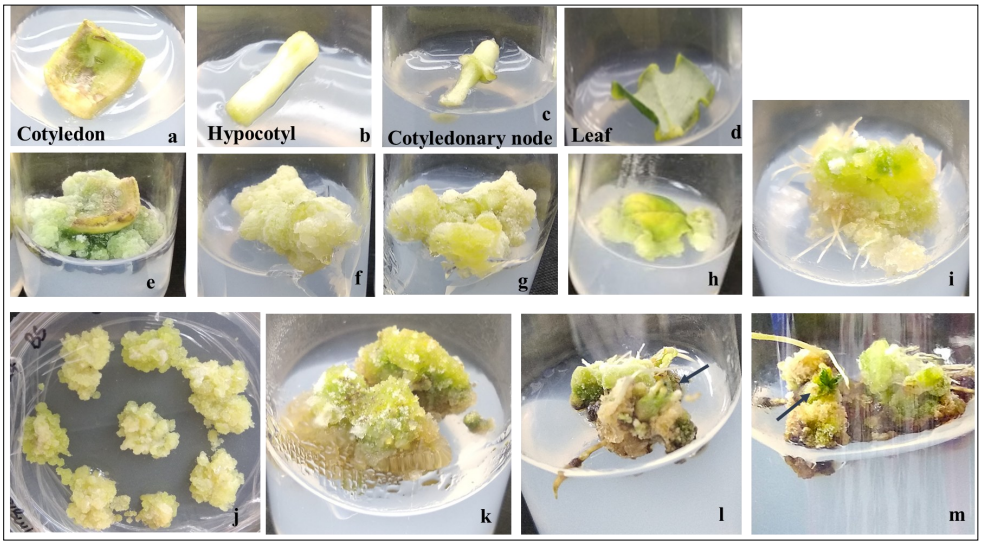



**Fig. 6.** Impact of different growth regulator for callus and shoot induction; a-d) after one-week different explants in the callus induction medium; e-h) at the end of three weeks; i) Roots emerged from the callus; j) Subculturing led to the proliferation of friable callus; k) powdery white layer formed above the callus in the shoot induction medium l) embryo like structure formed from callus after 3 week in the shoot induction (MS + ZT 1 mg/L + IAA 0.5 mg/L) m) shoots emerged from the callus.

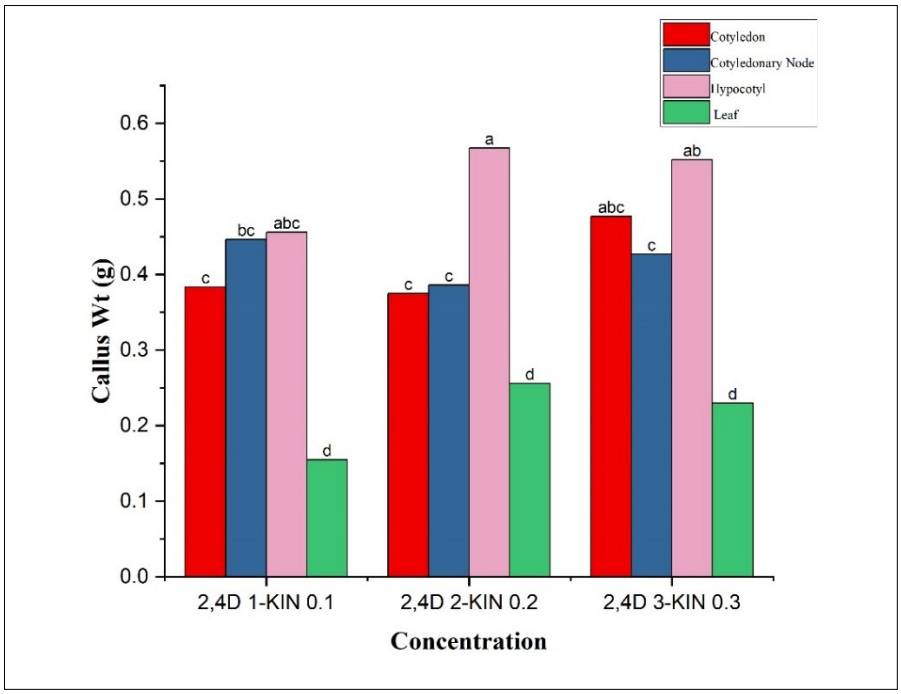
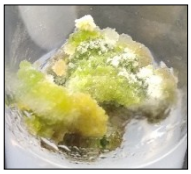
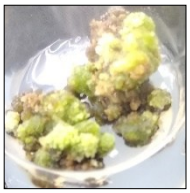



**Fig. 7.** Impact of 2,4-D and KIN combination in different explants.

**Table 2.** Effect of different growth regulators for shoot induction from callus

| S No. | Treatments (mg/L) | Shoot induction response | Morphology of the callus | |
|---|---|---|---|---|
| 1 | BAP 1.0 + IAA 0.5 | Root induced | White to green callus, powdery appearance on the surface |  |
| 2 | KIN 1.0 + IAA 0.5 | Root induced | White to green callus, powdery appearance on the surface |  |
| 3 | TDZ 1.0 + IAA 0.5 | No response | White to brown callus |  |
| 4 | ZT 1.0 + IAA 0.5 | Shoot and root induced | White to green callus, powdery appearance on the surface |  |
| 5 | BAP 2.0 + IAA 0.5 | Root induced | White to green callus, powdery appearance on the surface |  |
| 6 | KIN 2.0 + IAA 0.5 | Root induced | White to green callus, powdery appearance on the surface |  |
| 7 | TDZ 2.0 + IAA 0.5 | No response | White to green callus, browning |  |
| 8 | ZT 2.0 + IAA 0.5 | Root induced | White to green callus, powdery appearance on the surface |  |

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## Authors' contributions

KS conceptualized the research, participated in writing, reviewed the manuscript, supervised the work and approved the final draft. SB carried out the research, contributed to writing and assisted in illustrations. SR contributed to manuscript writing and review. KD participated in preparing illustrations. RKA assisted with illustrations and contributed to manuscript review. All authors have read and approved the final version of the manuscript.

## Compliance with ethical standards

**Conflict of interest:** The authors declare no conflict of interest.

**Ethical issues:** None

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