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Manu A.N
Indian Agricultural Research Institute
Anshul Sharma
Indian Agricultural Research Institute
Ramesh Kumar Yadav
Indian Agricultural Research Institute
J. K. Ranjan
Indian Agricultural Research Institute
Krishan Kumar
ICAR-Indian Institute of Maize Research
G. P. Mishra
Indian Agricultural Research Institute
Reeta Bhatia
Indian Agricultural Research Institute
Deepak Singh Bisht
National institute of plant biotechnology
Abhishek Jha
ICAR-Indian Institute of Maize Research
Reshav Naik
Indian Agricultural Research Institute
Bhargav S Kiran
Indian Agricultural Research Institute
Suman Lata
sumanlata3@gmail.com

Indian Agricultural Research Institute

Research Article

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Establishment of an efficient callus induction and in vitro plant regeneration protocol in okra (*Abelmoschus esculentus*(L.) Moench)

Manu A.N.[1], Anshul Sharma[1], Ramesh Kumar Yadav[1], J. K. Ranjan[1], Krishan Kumar[2], G. P. Mishra[3],Reeta Bhatia[4], Deepak Singh Bisht[5], Abhishek Jha[2], Reshav Naik[1], Bhargav S. Kiran[1], and Suman Lata[1]*

[1]Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi, India

[2]Delhi Unit Office, ICAR - Indian Institute of Maize Research, Pusa Campus, New Delhi, India

[3]Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi, India

[4]Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, New Delhi, India

[5]ICAR- National Institute of Plant Biotechnology, New Delhi, India

*Corresponding author: sumanlata3@gmail.com

Suman Lata, Division of Vegetable Science, ICAR-Indian Agricultural Research Institute, New Delhi, India

Manu A. N. and Anshul Sharma have equal contributions and share first authorship

Keywords Callus induction; Hypocotyl;Cotyledon;Somatic Embryogenesis; Regeneration;Okra;*Abelmoschus esculentus*

Abbreviations

AC: Activated Charcoal
BAP: 6-Benzylaminopurine
IBA: Indole-3-butyric Acid
KIN: Kinetin
MS: Murashige and Skoog
NAA: α-Naphthalene Acetic Acid
TDZ: Thidiazuron

Abstract

In the present investigation, a reproducible in vitro regeneration protocol was established for three okra cultivars—*Pusa Sawani, Pusa Bhindi-5* and *Pusa Lal Bhindi-1*-using hypocotyl and cotyledonary leaf explants. The explants were cultured on Murashige and Skoog medium (MS) comprising various auxin-cytokinin concentrations. MS mediumcontaining 2.0 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L α-Naphthalene acetic acid (NAA) and 200 mg/L activated charcoal (AC) was the most responsive,showing a mean organogenic callus induction frequency of 73.79% from hypocotyl explants across all three cultivars. This was followed by MS medium having1.0 mg/LBAP, 0.5 mg/L NAA,200 mg/L AC (66.50%) and 2.0 mg/L Zeatin,0.2 mg/L NAA,200 mg/LAC (65.29%). Cotyledonary leaf explants exhibiteda maximum callus induction frequency of 66.73% under the same hormonal combination. The lowest callus induction (23.18–24.28%) was observed on MS medium containing1.0 TDZ, 0.1 NAA,200 AC for both theexplants.The highest shoot induction (71.17%) with the earliest shoot bud emergence (13.25 days) was achieved on MS medium augmented with 2.0mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC. Optimal rooting was obtained on MS medium containing 0.2 mg/L NAA and 200 mg/L AC, resulting in 91.14% rooting, the earliest root initiation (14.82 days), and the longest roots (4.96 cm) in cultivar Pusa Lal Bhindi-1. As per our information, this is the first report detailing a robust and reproducible in vitro callus induction and regeneration protocol for the okra cultivars Pusa Sawani, Pusa Bhindi-5 and Pusa Lal Bhindi-1.

Introduction

Okra (*Abelmoschus esculentus* [L.] Moench; 2n=2x=130), also known as "Bhindi" or "lady finger,"belonging to the Malvaceae family is a traditional vegetable crop of significant commercial value.It holds considerable commercial value as a summer vegetable and is predominantly cultivated in tropical andsubtropicalareas globally. India is the leading producer of okra, contributing approximately 63% of the global production, with an area of 5.49 lakh hectares and a total production of 7.15 million tonnes (MT) (FAOSTAT-2023). Okra is known for its nutritional richness, containing vitamins A and C, dietary fibre, protein, carotenoids, and essential minerals such

as zinc, calcium, potassium, magnesium, and iodine, which contribute to its health benefits and commercial importance (Nathoo et al. 2025).The genus *Abelmoschus* exhibits considerable diversity in chromosome numbers and ploidy levels among species. The cultivated species, *A. esculentus* (2n = 130), is considered a natural amphidiploid species. *A. angulosus*has the lowest chromosome count is (2n=56), whereas while *A. manihot* var. *caillei* has the maximum(almost 200). In *A.esculentus,* chromosome numbers like 2n = 72, 108, 120, 132 and 144 follow a normal polyploid sequencebased on a basic chromosome number of n = 12. Despite extensive diversity, the precise origin of okra remains controversial, with proposed centres including Africa, the Upper Nile region, and India.Okra cultivation is severely constrained by various biotic and abiotic stresses, with viral infections being the most prominent biotic stress limiting production. Two major Begomoviruses affecting okra are the okra yellow vein mosaic virus (YVMV) and the okra enation leaf curl virus (ELCV) (Venkataravanappa et al. 2013). Although wild *Abelmoschus* species possess strong resistance to these viruses, their utilization in breeding programmes is limited due to both pre-and post-zygotic barriers (Badiger et al. 2023; Santhiya et al. 2022, 2025).Furthermore, the lack of linked polymorphic molecular markers has restricted the application of marker-assisted selection, while advanced breeding approaches such as genetic transformation, regeneration systems, and genome editing remain under-exploited in okra. (Lata et al. 2021; Yadav et al. 2025).

To date, limited research has been conducted on in vitro plant multiplication and regenerationin okra using explants for instanceapical shoots, cotyledons, cotyledonary nodes, and hypocotyl through direct and indirect organogenesis (Lata et al., 2025). Cotyledon and cotyledonary node were used as explants for demonstrating direct shoot regeneration in okra (Mangat and Roy 1986); while Roy and Mangat (1989) achieved plant regeneration via callus derived from cotyledonary nodes. Regeneration through somatic embryogenesis from suspension culturewas documented by Ganesan et al. (2007) a meristem culture protocol for large-scale production of disease-free plants was developed by Anisuzzaman et al. (2008). Subsequently, technique for indirect shoot organogenesis from leaf discs and hypocotyl explants through callus phase was then optimized by Anisuzzaman et al. (2010).Additional studies have reported indirect regeneration utilizing various explants and hormone combinations (Mallela et al., 2009; Irshad et al., 2017; Rizwan et al., 2020; Bensalem et al. 2023).Direct regeneration using shoot apices has also been documented (Dhande et al. 2012). Kiran et al. (2024) reported direct regeneration using epicotyl and embryo explants in wild species, interspecific hybrids and cultivated genotypes. Despite these efforts, reliable and reproducible indirect regeneration protocols remain limited for elite okra cultivars. Therefore, in this study,an indirect regeneration procedure was optimized for three widely cultivated and popular okra varieties —*Pusa Sawani, Pusa Bhindi-5*, and *Pusa Lal Bhindi-1*for which such protocols have not been previously reported. The development of a reproducible regeneration protocol is expected to facilitategenetic improvement of okra through genetic engineering and genome editing.Creating a robust regeneration protocol is essential for enabling improvement of okra through genetic transformation and genome editing.

Materials and methods

Plant material and seed sterilization

Three okra varieties i.e. *Pusa Sawani, Pusa Bhindi-5* and *Pusa Lal Bhindi-1,* developed and released by Division of Vegetable Science, Indian Agricultural Research Institute (IARI), New Delhi, were chosen for the study. Surface sterilization was performed following Kiran et al (2024). Briefly, seeds of all three varieties were treated with 1% carbendazim for 10 min and followed by five or six rinses with autoclaved distilled water in a laminar airflow cabinet.Seeds were then treated with 70% ethanol for 5 min andsoaked in 0.1% $HgCl_2$ solution for another 5 min. The seeds were then gently cleaned five or six times using autoclaved distilled water and allowed to air dry for eight to ten minutes inside a laminar airflow.Murashige and Skoog (MS) medium, with vitamins (Murashige and Skoog 1962) (HiMedia, India)was employed to inoculate the surface-sterilized seeds. In all experiments, MS medium was augmented with 30 g/L sucrose (CDH, India) and 3.0 g/L gelrite (Duchefa Biochemie, Netherlands). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 30 min.Subsequently, seeds were placed in a culture room for germination at a temperature of 26 ± 2 °C, under a 16/8 hour (light/dark) photoperiod with a light intensity of 50 µmol m−2 s−1, for a duration of 2-3 weeks.

Callus induction

Hypocotyland cotyledonary leavesexplantswereaseptically obtained from 14-day-old seedlings of each okra variety (Fig.1a). These hypocotyls were cultured on MS media with vitamins (Murashige and Skoog 1962) augmented with 200 mg/L activated charcoal (HiMedia, India) for callus induction in Petri dishes. The medium was supplemented with diverse concentrations of cytokinins and auxins. Cytokinin sources included 6-benzylaminopurine (BAP; HiMedia, India) at 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L; kinetin (KIN) at 1.0 and 1.5 mg/L;

zeatin (HiMedia, India) at 1.0, 1.5, 2.0, 2.5, and 3.0 mg/L; and thidiazuron (TDZ) at 1.0, 1.5, and 2.0 mg/L. These were combined with different auxin sources, including α-naphthaleneacetic acid (NAA; Titan Biotech, India) at 0.1, 0.2, 0.3, and 0.5 mg/L; indole-3-acetic acid (IAA; CDH, India) at 0.5 mg/L; and indole-3-butyric acid (IBA; CDH, India) at 0.2 mg/L (Table 1).These petri dishes were maintained at 26 ± 2 °C under dark conditions. Each treatmentwas conducted in three replications with approximately 45-50 hypocotyls explants per replication for each medium combination and variety. Following 5-6 weeks period of culture, the callus induction frequency was determined using the formula: *Callus induction frequency (%) = (Number of explants producing calli / Total number of explants inoculated) × 100.*

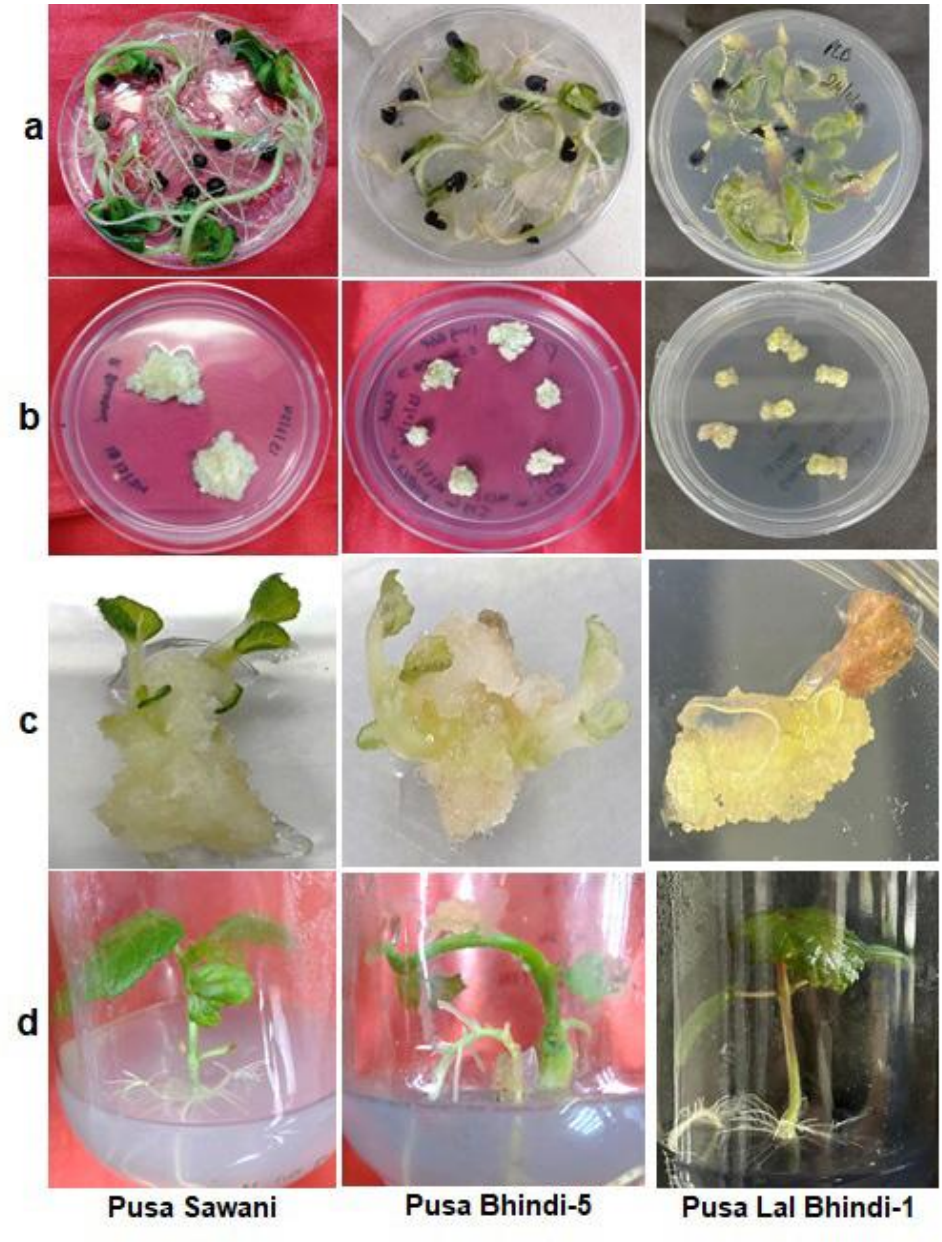

Fig. 1. Establishment of a callus-mediated in vitro regeneration protocol for the three okra varieties, namely Pusa Sawani, Pusa Bhindi-5 and Pusa Lal Bhindi-1. (a) In vitro germinated seedlings of okra. (b) Induction of embryogenic calli from hypocotyl explants on MS media added with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L activated charcoal (AC). (c) Shoot induction from hypocotyl-derived calli on MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC. (d) Root development in regenerated shoots on MS medium added with 0.2 mg/L NAA and 200 mg/L AC.

Table 1 Different auxin: cytokinin concentrations used for callusing in this study

Treatment No.	Growth regulator (mg/L)
C1	1.0 KIN + 0.1 NAA + 200 Activated charcoal
C2	1.5 KIN + 0.2 NAA + 200 Activated charcoal
C3	1.0 TDZ + 0.1 NAA + 200 Activated charcoal
C4	1.5 TDZ + 0.2 NAA + 200 Activated charcoal
C5	2.0 TDZ + 0.2 IBA + 200 Activated charcoal
C6	0.5 BAP + 0.1 NAA + 200 Activated charcoal
C7	1.0 BAP + 0.2 NAA + 200 Activated charcoal
C8	1.5 BAP + 0.5 IAA + 200 Activated charcoal
C9	1.0 BAP + 0.5 IAA + 200 Activated charcoal
C10	1.0 BAP + 0.5 NAA + 200 Activated charcoal
C11	3.0 BAP + 0.3 NAA + 200Activated charcoal
C12	1.5 BAP + 0.5 IBA+200 Activated charcoal
C13	2.0 BAP + 0.5 NAA+ 200 Activated charcoal
C14	1.5 Zeatin + 0.1 NAA + 200 Activated charcoal
C15	1.5 Zeatin + 0.2 IBA + 200 Activated charcoal
C16	2.0 Zeatin + 0.2 NAA + 200 Activated charcoal
C17	2.5 Zeatin + 0.2 IAA + 200 Activated charcoal
C18	3.0 Zeatin + 0.3 NAA + 200 Activated charcoal

Shoot regeneration

Embryogenic calli were divided into four equal-sized clumps and transferred to MS media with vitamins (Murashige and Skoog 1962), containingdifferent combinations of cytokinins and auxins for shoot initiation. The medium contained BAP at 1.0, and 2.0 mg/Lwith NAA at 0.2, and 0.5 mg/L or IAAat 0.5 mg/L.Zeatin was usedat 2.0, 3.0 mg/Lwith NAA at 0.2, and 0.3mg/L) andall media were supplemented with 200 mg/L activated charcoal and culture were maintained in petri dishesunder 16:8 h photoperiod conditions (Table 2).The number of shoots regenerated from the callus was recorded 30 days after subculturing. Each treatment consisted of three replications, with approximately 6-7 callus per replication for each media combination and variety. Following a culture period of two to four weeks, the number of shoot buds formed was recorded, and the regeneration frequency was determined using the following formula: *Regeneration frequency (%) = (Number of calli that produced shoots / Total number of calli inoculated) × 100.*

Table 2 Different auxin: cytokinin concentrations used for shooting in this study

Treatment No.	Medium	Growth Regulators (mg/L)
S1	MS	1.0 BAP + 0.2 NAA + 200 Activated Charcoal
S2	MS	1.0 BAP + 0.5 NAA + 200 Activated Charcoal
S3	MS	2.0 BAP + 0.2 NAA + 200 Activated Charcoal
S4	MS	2.0 BAP + 0.5 NAA + 200 Activated Charcoal
S5	MS	2.0 Zeatin + 0.2 NAA + 200 Activated charcoal
S6	MS	3.0 Zeatin + 0.3 NAA + 200 Activated charcoal

Rooting and acclimatization

To assess root induction, different auxin types and concentrations were tested.About 3–4 cm long regenerated shoots of okra were transferred to Murashige and Skoog (MS) medium augmented either with NAA at0.1,0.2, 0.3, 0.5 mg/L, or IBA at 0.2mg/L orIAA at 0.2mg/L (Table 3)and cultured in glass bottles for root initiation.Cultures were keptat 25 ± 2 °C, 16 h light/8 h dark photoperiod to promote root induction. Root emergence and growth were observed at regular intervals and data on days required for root initiation and root length prior to hardening were recorded. Plantlets with well-established roots were meticulously washed with water to eliminate any residual mediumand then transplanted into disposable plastic glasses containing peat, perlite and coco peat in 1:1:2 ratio. The glasses were covered with plastic sheets, which were perforated to allow proper aeration. The plantlets were maintained for 15 days under controlledenvironmental conditions at a temperature of 24 ± 2°C and 60% relative humidityfor acclimatization.

Table 3 Different auxin concentrations used for rooting in this study

Treatment No.	Media Composition (mg/L)
R1	MS + 0.1 NAA + 200 Activated Charcoal
R2	MS + 0.2 NAA + 200 Activated Charcoal
R3	MS + 0.3 NAA + 200 Activated Charcoal
R4	MS + 0.2 IBA + 200 Activated Charcoal
R5	MS + 0.2 IAA + 200 Activated Charcoal
R6	MS + 0.5 NAA + 200 Activated Charcoal

Statistical analysis: R software 4.5.0 (R Core Team, 2025) was used to analyse variation across all characters. A factorial completely randomized design (CRD) with three replications was employed for the analysis. Following the verification of assumptions regarding normal distribution and homogeneity of variances, deviation and statistical significance were significance were assessed using one-way analysis of variance (ANOVA). Statistics including mean, standard deviation (SD), and coefficient of variation (CV%), were calculated for all recorded traits.All graphical representations were generated using the R software.

Results:

Effect of hypocotyl and cotyledonary leaf explants on callus induction: Callus induction from hypocotyl explants varied significantly with growth regulator combinations, cultivars and their interactions (Fig. 2). Among the various treatments, the most effective callus induction, with a mean rate of 91.74%, was achieved using MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (treatment no. C13). This was followed by treatments with 1.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC, which resulted in an 83.65%

induction rate, and 2.0 mg/L Zeatin, 0.2 mg/L NAA, and 200 mg/L AC, which achieved an 82.43% induction rate. Conversely, the lowest callus induction rate, at 16.97%, was observed with the treatment comprising 1.0 mg/L TDZ, 0.1 mg/L NAA, and 200 mg/L AC (treatment no. C3) (Fig. 1b). L−1

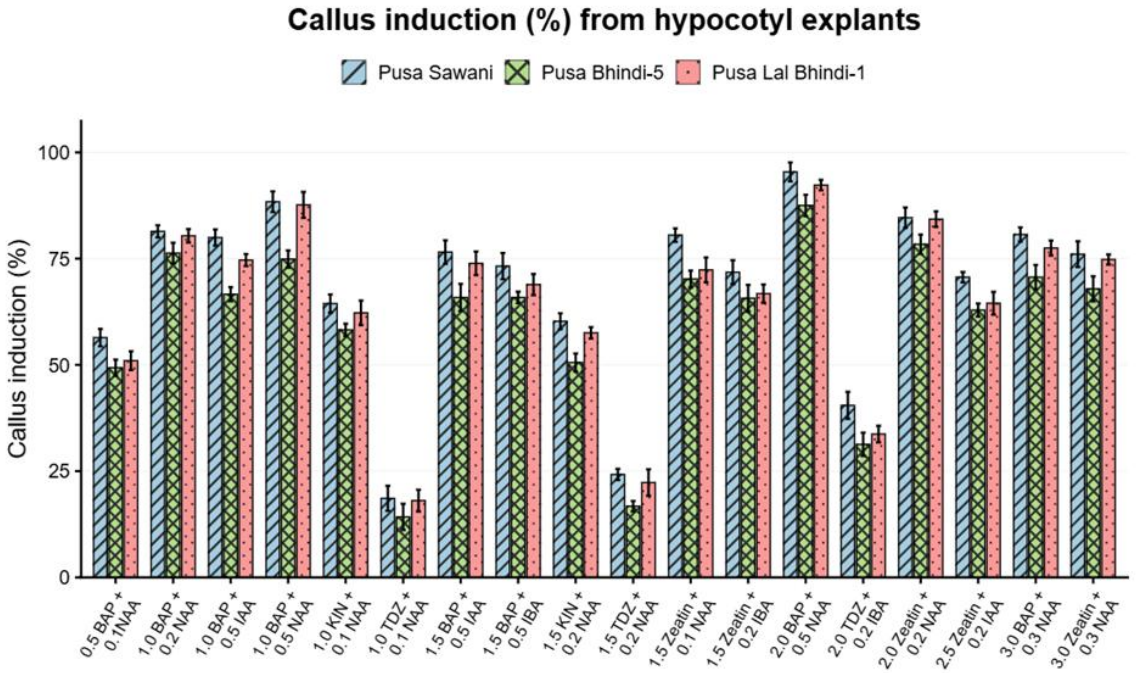

Fig. 2. Influence of various growth regulator combinations on callus induction (%) in hypocotyl explants. Values given are averages from three independent experiments.The error bar presents mean ± standard error.

Among the varieties, *Pusa Sawani*exhibited the highest mean callus induction response (67.98%), followed by *Pusa Lal Bhindi-1* (64.61%), whereas *Pusa Bhindi-5* recorded the lowest response (59.62%). The interaction between treatments and cultivars demonstrated that the highest callus induction rate (95.41%) was observed in Pusa Sawani cultured on MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC. Conversely, the lowest callus induction rate (14.24%) was recorded in Pusa Bhindi-5 with 1.0 mg/L TDZ, 0.1 mg/L NAA, and 200 mg/L AC.Statistical analysis showed that the critical difference (C.D.) values at the appropriate significance level for treatment, cultivar and their interaction were 1.86, 0.76 and 3.22, respectively, confirming that the observed differences were statistically significant.

Callus induction from cotyledonary leaf explants exhibited statistically significant variation among growth regulator combinations, cultivars and their interactions (Fig. 3). Among the treatments, the highest mean callus induction rate of 84.29% was achieved using MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (treatment no C13). This was followed by a callus induction rate of 66.50% with 1.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC, and 65.29% with 2.0 mg/L Zeatin, 0.2 mg/L NAA, and 200 mg/L AC. Conversely, the lowest callus induction rate of 24.28% was observed in MS medium added with 1.0 mg/L TDZ, 0.1 mg/L NAA, and 200 mg/L AC (treatment no C3).

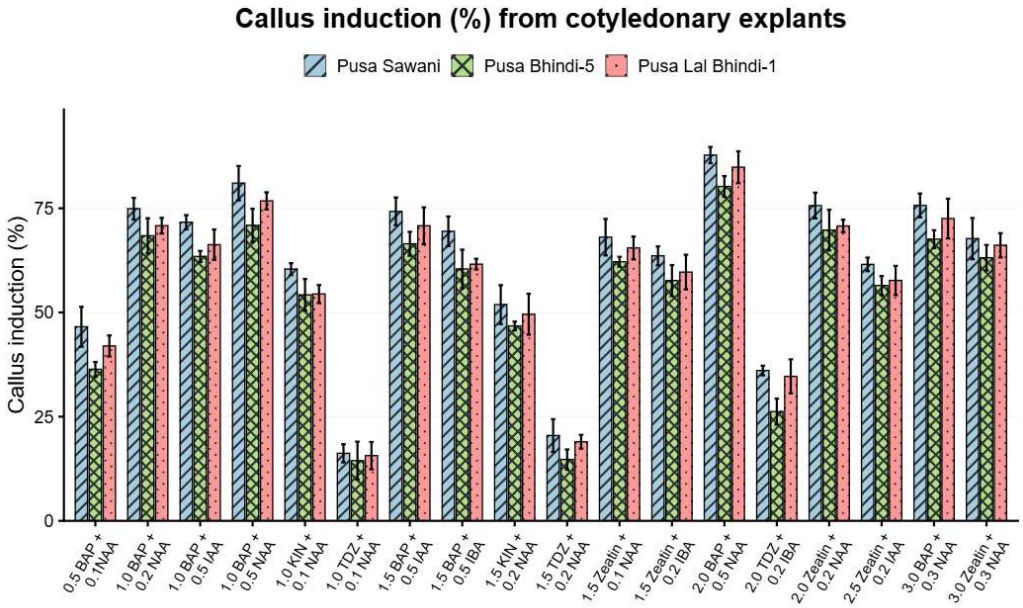

Fig. 3 Influence of various growth regulator combinations on callus induction (%) in cotyledonary leaf explant.Values given are averages from three independent experiments.The error bar presents mean ± standard error.

Among the cultivars, *Pusa Sawani* produced the highest mean callus induction response (61.28%), followed by *Pusa Lal Bhindi-1* (57.71%), whereas*Pusa Bhindi-5* recorded the lowest response (54.42%).Interaction analysisrevealed that the highest callus induction (87.77%) at 87.77%, was observed in Pusa Sawani when cultured on MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC. In contrast, the minimum callus induction (14.45%) was observed in *Pusa Bhindi-5* with with 1.0 mg/L TDZ, 0.1 mg/L NAA, and 200 mg/L AC. The critical difference (C.D.) values were 1.32 for treatments, 0.54 for cultivars and 2.28 for their interaction, indicating that all observed differences were statistically significant.Among the two explants used in this study, hypocotyl explants proved to be more responsive for callus induction, exhibiting a higher callus induction percentage than cotyledonary leaf explants.

Analysis of interactions indicated that the maximum rate of callus induction, at 87.77%, was found in Pusa Sawani when cultured on MS medium added with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC. Conversely, the lowest callus induction rate, at 14.45%, was recorded in Pusa Bhindi-5 with 1.0 mg/L TDZ, 0.1 mg/L NAA, and 200 mg/L AC.

Influence of treatments on days required for callus induction from hypocotyl and cotyledonary leaf explants:

The time required for callus initiation and culture establishment varied significantly with growth regulator combinations, cultivars, and their interactions (Fig 4 and 5). The minimum mean number of days for callus induction from hypocotyl explants (26.78 days) was recorded on MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (treatment no. C13), followed by 2.0 mg/L Zeatin, 0.2 mg/L NAA, and 200 mg/L AC (treatment no. C16) with 28.47 days and 1.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (treatment no. C10) with 28.79 days. In contrast, the maximum time for callus induction (35.47 days) was observed with 1.5 mg/L Zeatin, 0.2 mg/L IBA, 200 mg/L AC (treatment no. C15), closely followed by 1.5 mg/L TDZ, 0.2 mg/L NAA, and 200 mg/L AC (treatment no. C4) with 35.01 days (Fig 4).

Among the cultivars, *Pusa Lal Bhindi-1*exhibited fastest callus induction (31.68 days), followed by *Pusa Sawani* (32.84 days), while *Pusa Bhindi-5*required the longest time (33.50 days). The interaction between treatments and cultivars revealed that the shortest establishment period (25.36 days) was observed in *Pusa Lal Bhindi-1*cultured on MS medium added with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (treatment no. C13). Conversely, the longest period (37.00 days) observed in Pusa Bhindi-5 with 1.0 mg/L TDZ, 0.1 mg/L NAA, 200 mg/L AC (treatment no. C3). Statistical analysis showed critical difference (C.D.) values of 0.70 for treatments, 0.29 for cultivars and 1.21 for their interaction, confirming the significance of the variation observed.

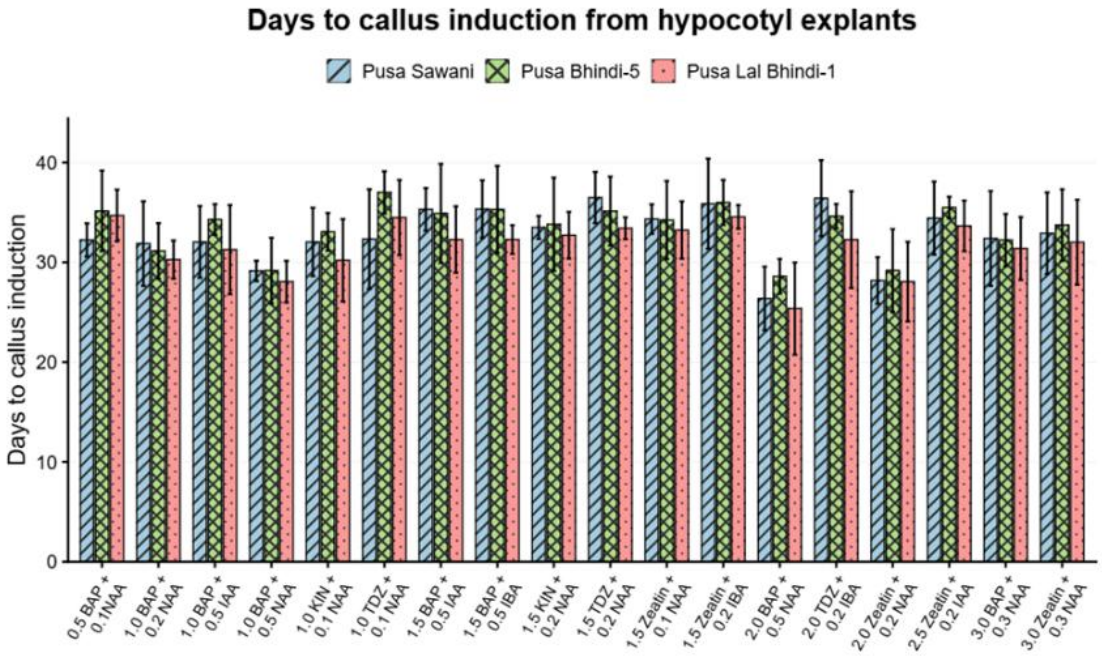

Fig.4 Influence of various growth regulator combinations on days required for callus induction from hypocotyl explants.The error bar presents mean ± standard error.

With cotyledonary leaf explants, the earliest callus initiation (28.46 days) was observed on MS medium added with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (treatment no. C13), then1.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC(29.88 days) and 2.0 mg/L Zeatin, 0.2 mg/L NAA, and 200 mg/L AC(31.08 days) (Fig 5). In contrast, the longest duration for callus initiation (37.64 days) was recorded with 1.0 mg/L TDZ, 0.1 mg/L NAA, and 200 mg/L AC(treatment no. C3).

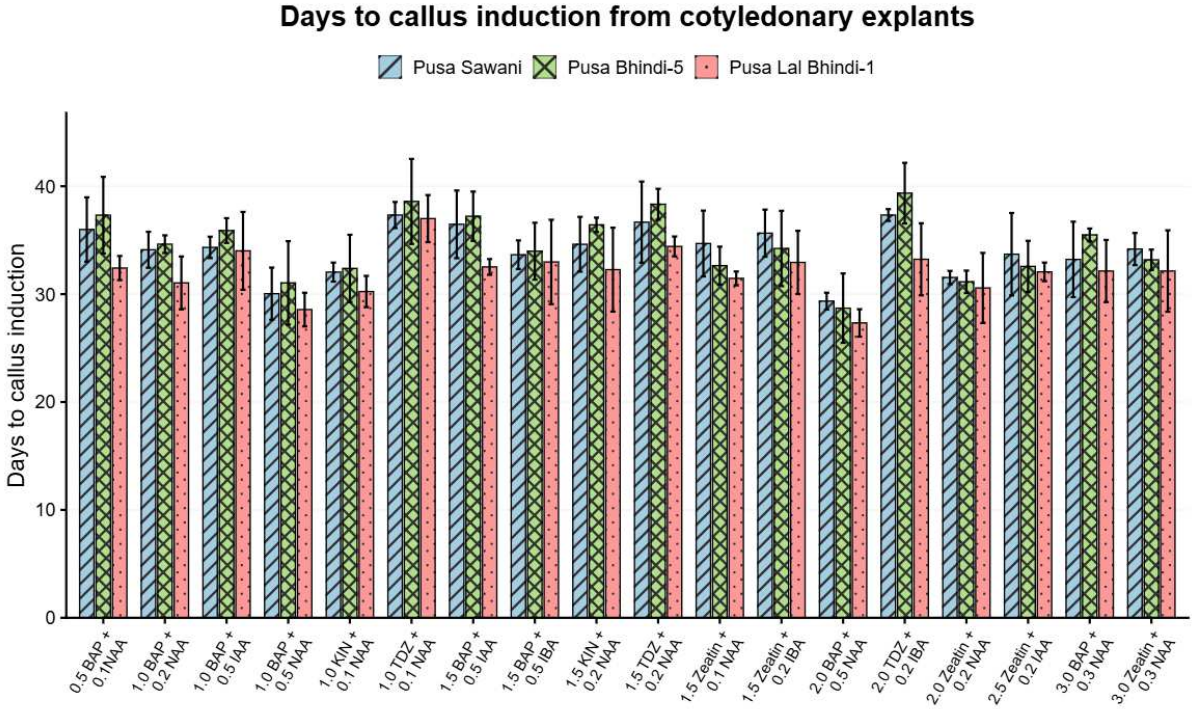

Fig. 5 Influence of various growth regulator combinations on days required for callus induction from cotyledonary leaf explants.The error bar presents mean ± standard error.

Across cultivars, Pusa Lal Bhindi-1 exhibited the fastest response, with a mean callus initiation time of 32.07 days, followed by *Pusa Sawani* (34.16 days), whereas*Pusa Bhindi-5*required the longest time (34.62 days). Interaction analysis revealed that the minimum callus initiation time (27.33 days) was observed in Pusa Lal Bhindi-1 cultured on MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC. Conversely, the maximum callus initiation time (39.36 days) was recorded in Pusa Bhindi-5 with 2.0 mg/L TDZ, 0.2 mg/L IBA, and 200 mg/L AC. Statistical analysis confirmed that the observed differences were statistically significant, with critical difference (C.D.) values of 1.04 for treatments, 0.42 for cultivars, and 1.80 for treatment × cultivar interactions.

The induced callus was predominantly friable in nature, and exhibited higher regenerative potential, while compact callus was comparatively less responsive (Fig.13). The callus colour ranged from white to yellowish-white and greenish-white, reflecting different developmental stages from nascent to more advanced stages with potential photosynthetic activity. Microscopic observation of revealed globular and Torpedo stages of callus (Fig.13).

Shoot bud initiation from callus:

For *in vitro* shoot initiation, only calli derived from hypocotyl explants were used, as hypocotyls exhibited superior callus induction compared to cotyledonary leaf explants. The maximum mean shoot bud induction (71.17%) was recorded in MS medium added with 2.0 mg/L BAP, 0.5 mg/L NAA, 200 mg/L AC (S4), followed by 2.0 mg/L BAP, 0.2 mg/L NAA, 200 mg/L AC (S3) with 55.65%, and 2.0 mg/L Zeatin, 0.2 mg/L NAA, 200 mg/L AC (S5) with 55.87% (Fig. 6) from hypocotyl derived callus(Fig. 1c). The minimum mean shoot bud induction (50.24%) was obtained in 1.0 mg/L BAP, 0.2 mg/L NAA, and 200 mg/L AC (S1). Among the cultivars, Pusa Sawani recorded the highest mean shoot bud induction (68.41%), followed by Pusa Lal Bhindi-1(56.48%) and least by Pusa Bhindi-5 (43.87%). The interaction between treatments and cultivars revealed that the highest shoot bud induction (82.58%) was observed in Pusa Sawani under 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (S4), whereas the lowest (39.18%) occurred in Pusa Bhindi-5 under 1.0 mg/L BAP, 0.2 mg/L NAA, 200 mg/L AC (S1). Statistical analysis showed that the differences among treatments (C.D. = 1.54), cultivars (C.D. = 1.09) and their interaction (C.D. = 2.67) were significant.

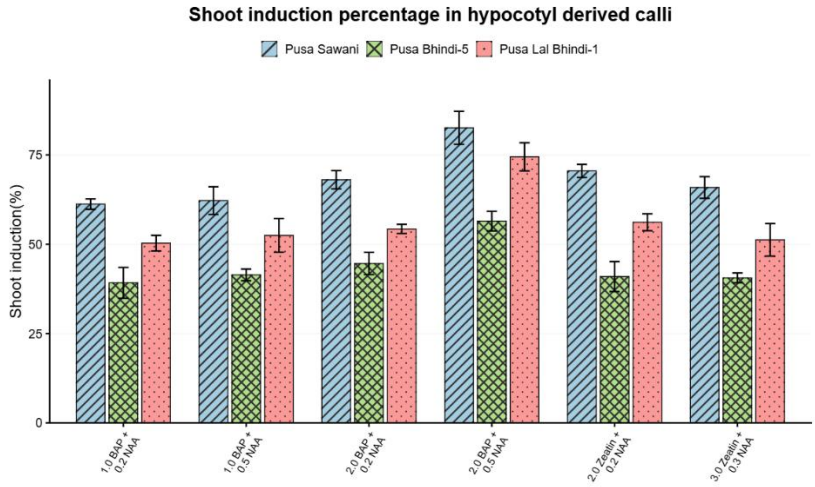

Fig. 6 Influence of various growth regulator combinations on shoot bud induction in hypocotyls-derived callus clump. The error bar presents mean ± standard error.

The maximum mean shoot bud induction (71.17%) from hypocotyl-derived callus was recorded on MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, 200 mg/L activated charcoal (AC) (S4), followed by 2.0 mg/L BAP, 0.2 mg/L NAA, 200 mg/L AC (55.65%) (S3) and 2.0 mg/L zeatin, 0.2 mg/L NAA, 200 mg/L AC (55.87%) (S5) (Fig. 6). In contrast, the minimum mean shoot bud induction (50.24%) was observed with 1.0 mg/L BAP, 0.2 mg/L NAA, and 200 mg/L AC (S1).

The least mean number of days to shoot bud initiation (13.25 days) was recorded onMS medium added with 2.0 mg/L BAP, 0.5 mg/L NAA, 200 mg/L AC, followed by 2.0 mg/L Zeatin, 0.2 mg/L NAA, and 200 mg/L AC with 15.41 days, and 3.0 mg/L Zeatin, 0.3 mg/L NAA, and 200 mg/L AC with 16.74 days (Fig. 7). The maximum time (23.84 days) was found in 1.0 mg/L BAP, 0.2 mg/L NAA, and 200 mg/L AC. Among the cultivars, Pusa Lal Bhindi-1 exhibited the shortest time to shoot bud initiation (17.72 days), followed by Pusa Sawani (17.85 days),

whereas Pusa Bhindi-5 required the longest time (19.24 days). The interaction between treatments and cultivars revealed that the shortest shoot bud initiation period (12.50 days) was recorded in Pusa Lal Bhindi-1 under S4, whereas the longest period (24.80 days) was observed in Pusa Bhindi-5 under S1. Statistical analysis indicatedthat the critical difference (C.D.) values for treatment, cultivar and their interaction were 0.58, 0.41 and 1.01 days, respectively, confirming significant differences.

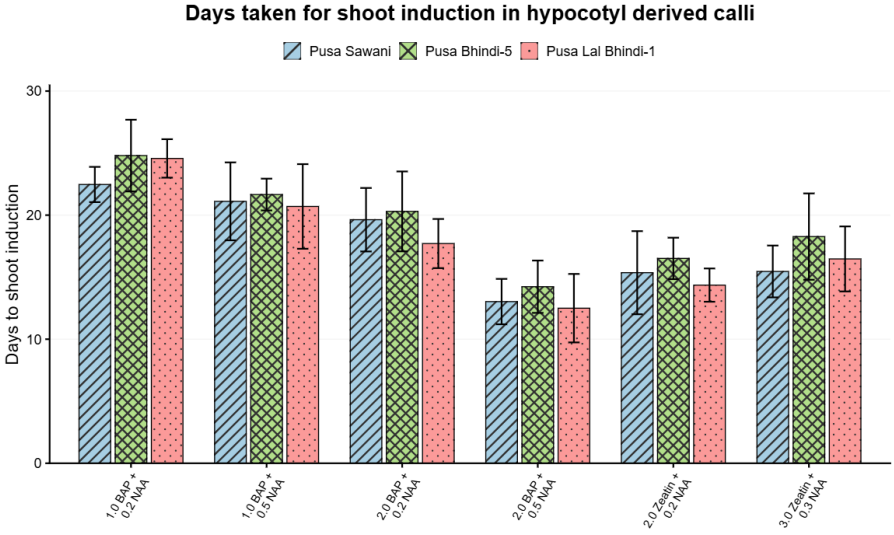

Fig. 7 Influence of various growth regulator combinations on number of days required for shoot bud induction in hypocotyls-derived callus clump. The error bar presents mean ± standard error.

The maximum mean shoot length (6.10 cm) was obtained in MS medium added with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (S4), followed by 2.0 mg/L Zeatin, 0.2 mg/L NAA, and 200 mg/L AC (S5) with 5.50 cm and 3.0 mg/L Zeatin, 0.3 mg/L NAA, and 200 mg/L AC (S6) with 5.33 cm (Fig. 8). The minimum mean shoot length (4.33 cm) was recorded in 1.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC (S2). Among the cultivars, Pusa Lal Bhindi-1 exhibited the longest shoots (5.84 cm), followed by Pusa Sawani (5.13 cm), while Pusa Bhindi5 recorded the shortest shoots (4.49 cm). In treatment × cultivar interactions, the longest shoots (7.13 cm) were observed in Pusa Lal Bhindi-1 under S4, whereas the shortest shoots (3.99 cm) occurred in Pusa Bhindi-5 under S2. Statistical analysis revealed critical difference (C.D.) values of 0.13, 0.09 and 0.23 for treatment, cultivar and interaction, respectively, confirming significant differences.

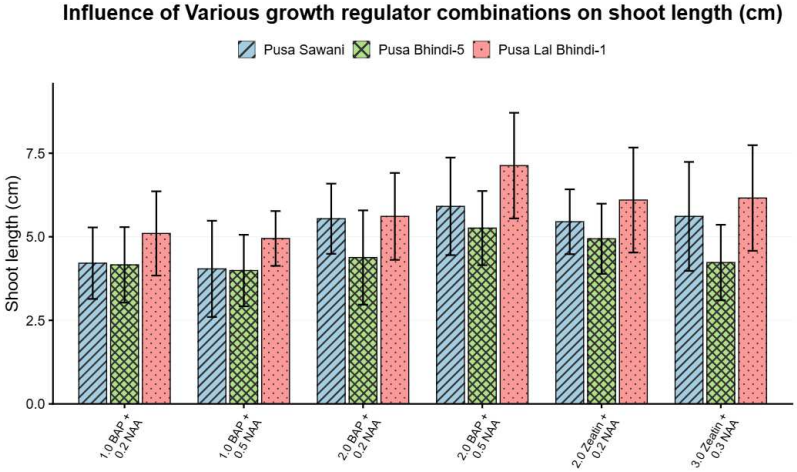

Fig. 8 Influence of various growth regulator combinations on the average shoot length of plantlets prior to hardening.The error bar presents mean ± standard error.

Rooting and acclimatization of plantlets

The highest mean rooting percentage (91.14%) was observed on MS medium augmented with 0.2 mg/L NAA and 200 mg/L AC (R2), followed by 0.3 mg/L NAA and 200 mg/L AC (83.90%) and 0.5 mg/L NAA and 200 mg/L AC (75.41%) (Fig.9)(Fig. 1d). The lowest mean rooting percentage (64.41%) was recorded with 0.2 mg/L IBA and 200 mg/L AC (R4).Among the cultivars, Pusa Lal Bhindi-1 showed the maximum average rooting response (79.42%), while Pusa Bhindi-5 recorded the lowest (70.64%). Statistical analysis revealed that differences among treatments, cultivars, and their interactions were significant, with critical differences (C.D.) of 1.44, 1.02 and 2.49, respectively.

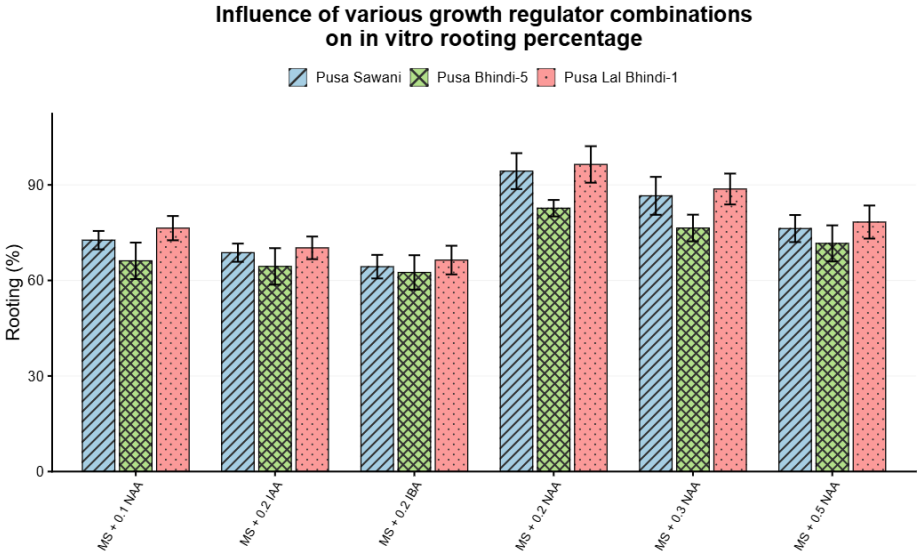

Fig. 9 Influence of various growth regulator combinations on in vitro rooting percentage.The error bar presents mean ± standard error.

The minimum number of days required for root development (16.45 days) was recorded in the cultivar Pusa Sawani, whereas the maximum (18.413 days) was recorded in the cultivar Pusa Bhindi-5.The earliest rooting response was obtained on MS medium augmented with 0.2 mg/L NAA and 200 mg/L AC (R2), which recorded the lowest mean value of 14.82 days, followed by 0.3 mg/L NAA and 200 mg/L AC (17.10 days) and 0.5 mg/L NAA and 200 mg/L AC (17.40 days) (Fig. 10). In contrast, the maximum number of days for root initiation (21.67 days) was found on MS medium containing 0.1 mg/L NAA and 200 mg/L AC (R1). The treatment × cultivar interaction showed that Pusa Lal Bhindi-1 exhibited the minimum average number of days to rooting (16.73 days), while Pusa Bhindi-5 required the maximum (19.16 days). Statistical analysis confirmed that the differences among treatments, cultivars, and their interactions were significant, with critical difference (C.D.) values of 0.49, 0.35 and 0.85 days, respectively.

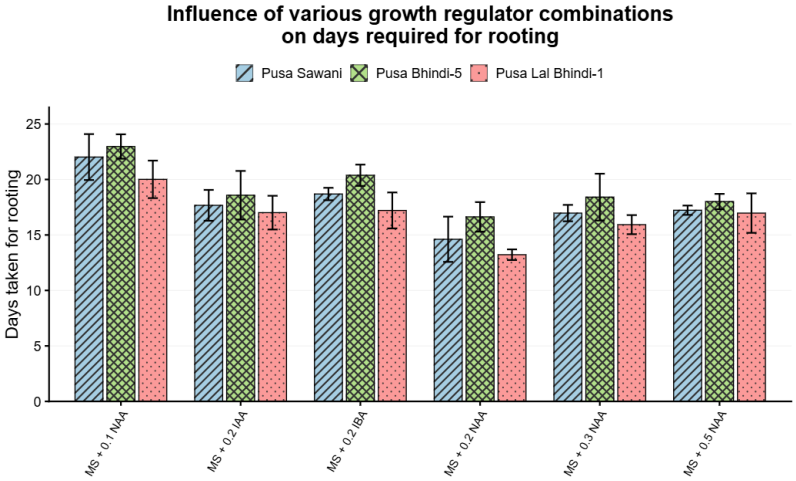

Fig. 10 Influence of various growth regulator combinations on days required for rooting.The error bar presents mean ± standard error.

The maximum mean root length (4.96 cm) was found on MS medium added with 0.2 mg/L NAA and 200 mg/L AC (R2), followed by 0.3 mg/L NAA and 200 mg/L AC (4.56 cm) (Fig.11). In contrast, the smallest root length (2.41 cm) was observed with 0.2 mg/L IBA and 200 mg/L AC (R4). Treatments containing IAA (2.71 cm) and higher concentrations of NAA (0.5 mg/L; 2.45 cm) also resulted in comparatively shorter roots. Among the cultivars, Pusa Sawani (3.46 cm) and Pusa Lal Bhindi-1 (3.45 cm) exhibited greater mean root length, while Pusa Bhindi-5 recorded the lowest (2.92 cm). Statistical analysis showed that differences among treatments, cultivars and their interactions were significant, with critical difference (C.D.) values of 0.18, 0.13 and 0.32, respectively. Well-rooted plantlets were successfully acclimatized following transfer from in vitro conditions to the National PhytotronFacility (IARI, New Delhi)(Fig. 12a). Morphologically, the hardened plants maintained healthy foliage, robust stems and active root systems, confirming the effectiveness of the hardening process in ensuring successful establishment (Fig. 12b). The entire okra regeneration protocol with days required at each stage is depicted in fig. 13.

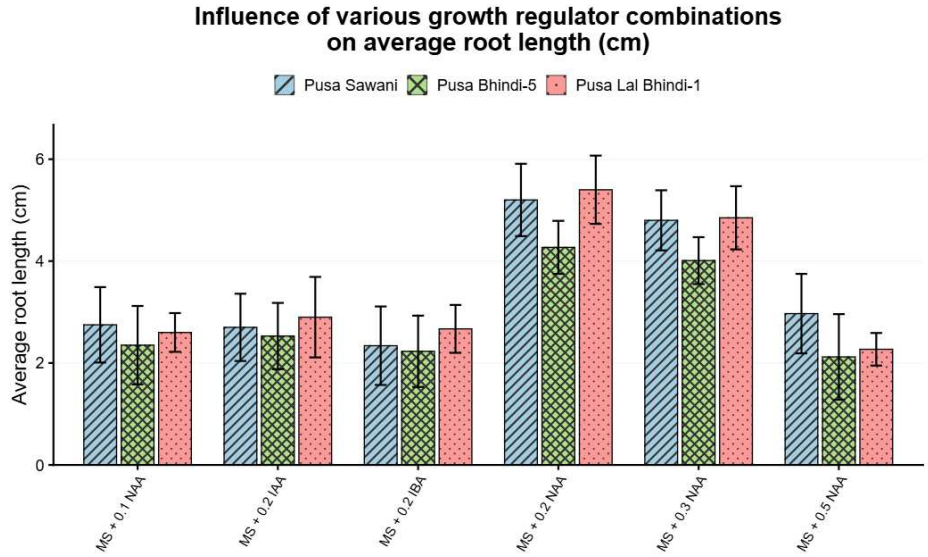

Fig. 11 Influence of various growth regulator combinations on the average root length of plantlets prior to hardening.The error bar presents mean ± standard error.

Fig. 12 Rooting and acclimatization of in vitro regenerated okra plantlets: (a) well-rooted regenerated plantlets; (b) successful acclimatization of regenerated plants under ex vitro conditions.

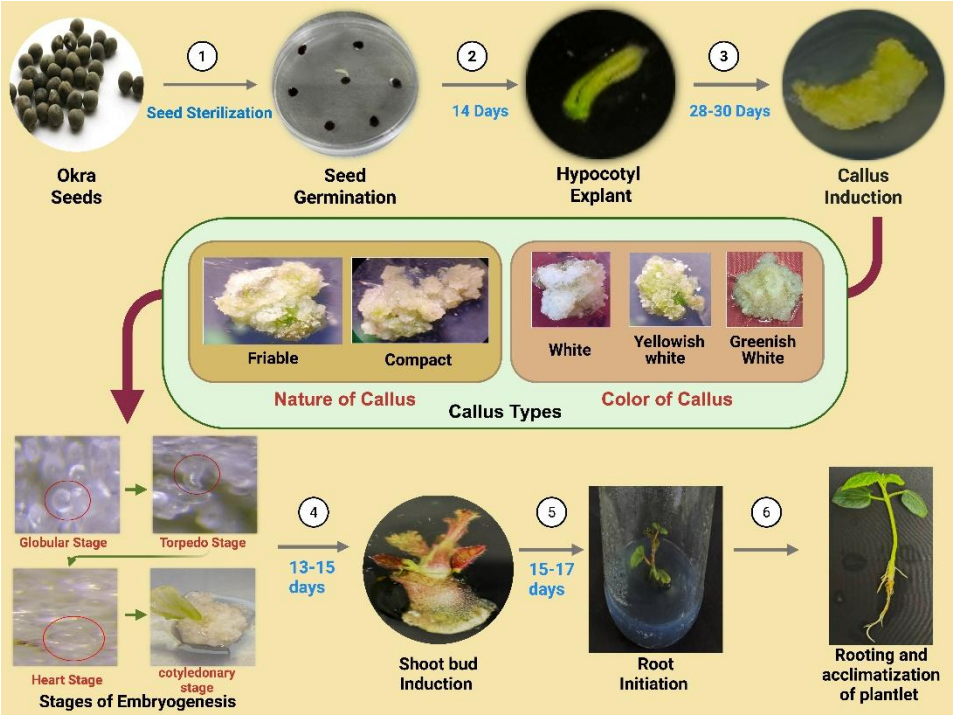

Fig. 13 Schematic representation showing okra regenration protocol.

Discussion

In vitro culture has emerged as a crucial methodology for the enhancement of crops and vegetables, throughits application in genetic transformation, generationof transgenic plants, development of haploids, clonal regeneration and genome editing (Kumar et al., 2022). Micropropagation also plays a crucial role in maintaining and propagating specific breeding lines in okra. However, okra is considered a recalcitrant species for tissue culture, primarily because of high phenolic exudation, which leads to browning and tissue necrosis (Irshad et al., 2017), formation of compact and non-embryogenic callus (Ganesan et al., 2007; Anisuzzaman et al., 2010) and browning of calli (Elkonin and Pakhomova2000). Up until now, there have been only a few studies conducted on the indirect regeneration of okra (Anisuzzaman et al., 2008; Mallela et al., 2009; Irshad et al., 2017; Rizwan et al., 2020; Bensalem et al. 2023).However,anefficient and reproduciblecallus-mediated regeneration protocol for okra is lacking.Therefore, optimization ofindirect regeneration protocols is essential for enablinggenetic transformation and improvement of economically important traits in okra.The efficiency of callus induction and regeneration is determined by various factors, including the chemical composition of the culture medium, the type of explant, the plant genotype, and the culture conditions.Among the explants evaluated, hypocotyls exhibited significantly higher callus induction rates than cotyledonary leaves. Among the cytokinins evaluated-BAP, kinetin, zeatin, and TDZ. BAP has shown maximum efficiency for callus induction, followed by zeatin, kinetin, and TDZ.Hypocotyl explants cultured on MS medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 mg/L AC exhibited the highest callus induction frequency (91.74%). The superior responsiveness of hypocotyls may be attributed to their juvenile meristematic cells, higher endogenous cytokinin levels and reduced lignification, in agreement with earlier reports (Roy and Mangat, 1989; Mallela et al., 2009). The optimized BAP:NAA ratio likely provided a favourable hormonal balance for dedifferentiation and proliferation without inducing premature organogenesis (George et al., 2008). Activated charcoal plays a crucial role in improving callus quality by adsorbing phenolic exudates and maintaining a stable hormonal environment (Irshad et al., 2017). The earliest callus formation was observed in hypocotyl explants (26.78 days) than cotyledonary leaves (28.46 days). In comparison, Anisuzzaman et al. (2008) reported callus initiation within just 7–10 days using NAA and TDZ, while Kabir et al. (2008) observed callus formation after approximately three weeks, which aligns with the present findings.Interestingly, the same hormone combination that produced the highest callus induction also resulted in the highest shoot initiation when cultured under a 16:8 h photoperiod. Hypocotyl-derived callus cultured on MS medium added with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 AC exhibited the highest shoot bud induction (71.17%), with Pusa Sawani recording the maximum cultivar-specific response (82.58%).

Notably, the identical hormonal combination that facilitated the highest callus induction also yielded the most significant shoot initiation when cultured under a 16:8 h photoperiod. Hypocotyl-derived callus cultured on MS

medium augmented with 2.0 mg/L BAP, 0.5 mg/L NAA, and 200 AC demonstrated the highest shoot bud induction rate (71.17%), with the Pusa Sawani cultivar exhibiting the maximum specific response (82.58%).

The effectiveness of BAP in shoot induction can be attributed to its cytokinin activity, stability and strong receptor affinity compared to kinetin or TDZ (Mallela et al., 2009). The earliest shoot initiation was observed at 13.25 days under the optimal treatment. Pusa Lal Bhindi-1 produced the longest shoots (7.13cm), indicating genotype--dependent response. Dhande et al. (2012) reported 98.5% shoot regeneration with shoot lengths of approximately 3.5 cm using apical shoot explants cultured on MS medium augmented with 1.0 mg/L IBA and 0.5 mg/L NAA. In contrast, Bensalem et al. (2023) reported notably longer shoots (10.28 cm) using TDZ and BAP combinations on Gamborg B5 medium, indicating the influence of both hormone composition and basal medium on shoot elongation.Both NAA and IBA concentrations significantly influenced in vitro rooting and root quality. In vitro rooting using MS medium added with 0.2 mg/L NAA and 200 mg/L activated charcoal resulted in in the highest rooting frequency(91.52%), the earliest root initiation (14.43 days) and vigorous root morphology. The superior rooting response at this auxin concentration can be attributed to the optimal stimulation of root primordia formation, while activated charcoal likely enhanced rooting by adsorbing inhibitory substances and maintaining a favourable medium environment. These results are consistent with previous reports in okra. For instance, Dhande et al. (2012) achieved effective rooting using 0.5 mg/L IAA with 1 g/L activated charcoal, while Rizwan et al. (2020) reported up to 83% rooting using 1.0 mg/L IBA with 200 mg/L activated charcoal. Similarly, Irshad et al. (2017) observed strong root systems using 2 mg/L IBA combined with 200 mg/L activated charcoal yielded prior to hardening. The findings collectively highlight the effectiveness of activated charcoal in enhancing rooting efficiency, aligning with the observations of Rattan et al. (2020). Akogo et al. (1996) and Anisuzzaman et al. (2008) documented root emergence within two weeks on MS medium, whereas Kabir et al. (2016) reported rooting as early as 9.7 days using 2.46 µM IBA.In contrast, Mallela et al. (2009) observed root induction between 18 and 24 days on NAA-containing media, which stillfalls within with an early rooting window.The observation that maximum callus and shoot regenerationoccurred under the same hormone combination underscores the critical importance of this specific auxin-cytokinin balance in orchestrating both cellular proliferation and organogenesis in okra. This hormonal regime effectively promoted dedifferentiation, where explant cells revert to a totipotent callus state, followed by redifferentiation into organized shoot structures—key phases for successful in vitro regeneration. The synergistic interaction between auxins and cytokinins within this combination likely establishes an optimal physiological environment that promotes cell division, elongation, and differentiation, consistent with well-established plant tissue culture paradigms where the auxin-to-cytokinin ratio is a key determinant of morphogenic responses.

Conclusion

This study successfully developed an effective protocol for in vitro culture and subsequent regeneration in okra (*Abelmoschus esculentus*), providing valuable insights into the effects of various growth regulators at various stages of plant tissue culture. Among the explants, the hypocotyl was identified as the most responsive for callus induction, achieving the highest callusing percentage, whereas, the cotyledonary leaf showed moderate callusing potential. The study demonstrated that the MS medium added with 2.0 mg/L BAP, 0.2 mg/L NAA, and 200 mg/L activated charcoal was the most effective combination for callusinduction across all three cultivars. Callus induction improved significantly with increasing levels of BAP, up to 2.0 mg/L, which also reduced the days required for callus initiation. In vitro shoot regeneration was most effective when BAP was used as the cytokinin source, with optimal results achieved on MS medium containing 2.0 mg/L BAP, 0.2 mg/L NAA, and 200 mg/L activated charcoal. This hormone combination also led to the shortest time to shoot initiation and the maximum shoot length before hardening. For in vitro rooting, MS medium augmented with 0.2 mg/L NAA was found to be optimal, yielding superior root length, volume, and weight, while minimizing the time required for root development. Overall, this study enhances the understanding of tissue culture responses in okra and provides a robust and reproducible regeneration framework.The established protocol holds significant potential for future applications in genetic transformation, genome editing and crop improvement of this economically important yet underexploredspecies.

Data availability statement: All data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions Conception and design of the experiments: **SL, RKY**; carried out the experiments: **MAN, AS**; Data analysis: **MAN, AS, JKR,AJ, RN, SBK**; Contributed reagents/materials/analysis tools: **SL, RKY**; Wrote the paper and edited the manuscript: **SL, KK,GPM, RB, DSB**.

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Conflict of interest: The authors state that they have no conflicts of interest.

Ethics declaration: This research does not include any studies involving human or animal subjects conducted by the authors.

Ethics and consent to participate:

Ethical approval was not required for this research as it involved cultivated released varieties as plant materials. The collection of plant materials was done in accordance with institutional guidelines and regulations. Permission to collect plant materials and carry out the experiment was granted by the relevant authorities.