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## DECIPHERING GENOTYPE AND EXPLANT SPECIFIC *IN VITRO* SHOOT REGENERATION IN CUSTARD APPLE (*ANNONA SQUAMOSA* L.)

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### ABSTRACT

Custard apple (*Annona squamosa* L.) is an important fruit crop in arid and semi-arid India, but conventional propagation methods are slow and inefficient, highlighting the need for alternative strategies to produce uniform and high-quality planting material. Although tissue culture is promising, custard apple is recalcitrant due to contamination, poor establishment, and low regeneration. This study aimed to standardize an *in vitro* shoot regeneration protocol by assessing genotype and explant specific responses to surface sterilization and growth regulator combinations. Nodal segments and shoot tips from three genotypes, Balanagar, Hybrid-12, and Phule Janaki were cultured on Murashige and Skoog medium supplemented with cytokinins and gibberellins. Balanagar exhibited superior shoot induction, earlier initiation, and greater shoot length on BAP and KIN, while shoot tips responded best to BAP and GA<sub>3</sub>. Rooting was limited by leaf abscission, indicating the need for further optimization for complete plantlet development.

**Key words:** *Annona squamosa* L., *In vitro*, Micropropagation, Shoot induction, Surface sterilization

### Introduction

Custard apple (*Annona squamosa* L.), also known as sugar apple or sweetsop, is a member of the Annonaceae family widely cultivated in arid and semiarid regions of India. Native to tropical America, it is valued for its delicious pulp, rich in carbohydrates, vitamins, and proteins, along with its medicinal properties, which include vermifugal, insecticidal, and therapeutic applications (Troup, 1975; Coronel, 1983, Mathew *et al.*, 2025). Extracts from *A. squamosa* leaves and seeds could help create novel antibiotics and treatments for cancer (Al-Nemari *et al.*, 2020, Hasan *et al.*, 2025). Despite its economic and nutritional importance, custard apple cultivation is constrained by low seed germination rates (30–40%), significant genetic variability due to cross-fertilization, and challenges in clonal propagation (George *et al.*, 1987; Oliveira *et al.*, 2008).

Traditional propagation methods, such as grafting and budding, offer some advantages but are slow and labor-

intensive, while techniques like air layering and cutting show limited success due to poor rooting (Rasai *et al.*, 1994; 1995). These limitations necessitate the development of alternative propagation strategies to meet the growing demand for uniform and high-quality planting material.

*In vitro* regeneration offers a promising solution by enabling rapid and efficient propagation of elite genotypes. However, custard apple is considered recalcitrant to tissue culture, facing challenges such as contamination, poor culture establishment, and low regeneration rates (Santana *et al.*, 2003; Oliveira *et al.*, 2007). To address these issues, the present study aims to develop a standardized *in vitro* shoot regeneration protocol for custard apple.

The research focuses on evaluating the response of various explants to different surface sterilization methods and combinations of growth regulators. Establishing a robust regeneration protocol is not only critical for large-

**Table 1:** Sterilants used for surface sterilization for explants.

Sterilant	Duration (min.)
HgCl <sub>2</sub> (0.1%, w/v)	5
	10
	15
NaClO (2%, w/v)	5
	10
	15

scale propagation but also serves as a foundation for genetic transformation studies. For example, traits such as improved shelf life could be achieved through genetic modification, including the silencing of genes involved in ethylene production. By overcoming the challenges associated with *in vitro* culture of custard apple, this study seeks to contribute to its sustainable cultivation and broader applications in horticulture and biotechnology.

## Materials and Methods

### Explant collection and surface sterilization

Nodal segments and shoot tips were collected from healthy, green mother plants of custard apple (*Annona squamosa* L.) maintained at the All India Coordinated Research Project (AICRP) on Arid Zone Fruits, Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, India. Explants were harvested primarily from secondary and tertiary branches using sterilized secateurs, wrapped in polythene sheets, and promptly transported to the laboratory to maintain tissue viability.

For surface sterilization, the explants were first washed under running tap water to remove surface debris, followed by immersion in Tween-20 solution for 10 minutes with constant shaking. The samples were then rinsed thoroughly with sterile distilled water. Subsequently, they were treated with 0.2% Bavistin and 0.05% streptomycin for 30–60 minutes at room temperature to eliminate fungal and bacterial contaminants. Final surface sterilization was performed inside a laminar airflow chamber using 2% sodium hypochlorite (NaClO) and 0.1% mercuric chloride (HgCl<sub>2</sub>) for durations standardized and detailed in Table 1. To mitigate phenolic leaching, the explants were immersed in an antioxidant solution containing 100 mg/L each of citric acid and ascorbic acid for 10 minutes.

### Culture conditions and experimental design

Sterilized nodal and shoot tip explants (2–3 cm in length) were cultured on Murashige and Skoog (MS) medium supplemented with 3% sucrose and 100 mg/L activated charcoal to reduce browning. Cultures were initially incubated in complete darkness to support establishment and then transferred to a growth room

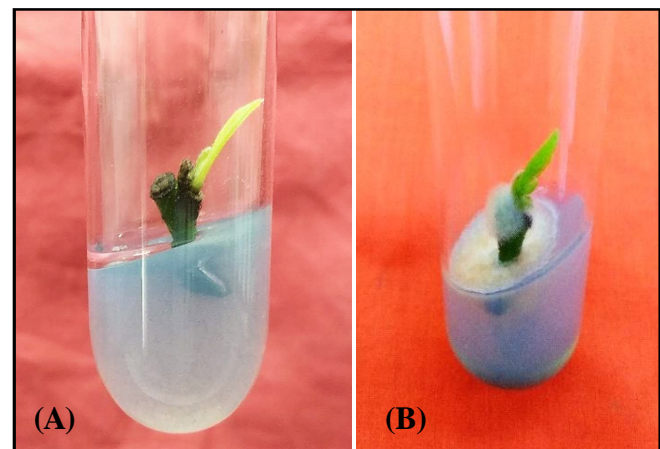
**Table 2:** Hormonal combinations used for shoot induction.

Explant	Hormonal Concentration
Node	BAP(0.4mg/l) + KIN(0.4mg/l)
	BAP(0.5mg/l) + KIN(0.5mg/l)
	BAP(0.6mg/l) + KIN(0.6mg/l)
	BAP(0.7mg/l) + KIN(0.7mg/l)
	BAP(0.8mg/l) + KIN(0.8mg/l)
Shoot tip	BAP(2.0mg/l) + GA <sub>3</sub> (0.5mg/l)
	BAP(4.0mg/l) + GA <sub>3</sub> (0.5mg/l)

maintained at 25 ± 2°C with a 16-hour light and 8-hour dark photoperiod. Shoot multiplication was carried out through subculturing at 15-day intervals using the same hormonal compositions. The entire experiment followed a factorial completely randomized design (FCRD) with three replications per treatment to ensure statistical validity.

### Culture media and shoot induction

Preliminary shoot induction trials were conducted using nodal segments from the Balanagar genotype. The explants were cultured on MS medium supplemented with different combinations of growth regulators. The combinations included BAP (0.5 mg/L) + KIN (0.5 mg/L) + IBA (1.0 mg/L) as per Abubacker and Deepalakshmi (2017), and further variants such as BAP (1.0 or 2.0 mg/L) + KIN (0.5 mg/L) + IBA (1.0 or 2.0 mg/L). An additional treatment with BAP (0.6 mg/L) + KIN (0.6 mg/L) was included based on the protocol by Farooq *et al.*, (2002). Hormone-free MS medium was used as a control. Observations on regeneration response were recorded 15 days after inoculation, comprising percent shooting, days to shoot initiation, and shoot length. To circumvent the need for surface sterilization, *in vitro* grown seedlings from the seeds were also utilized in the experiment.



**Fig. 1:** Effects of surface sterilization on *in vitro* culture of explants A: Contamination free shoot establishment of nodal explants. B: Fungal contamination of the nodal explants.

**Table 3:** Results of surface sterilization of nodal explants (cv. Balanagar).

Sterilant	Dm	CE	C	SA	CT
HgCl <sub>2</sub> (0.1%, w/v)	5	4	26.67	73.33	Fungal
	10	1	6.67	93.33	Fungal
	15	Browning	-	-	-
NaOCl (2%, w/v)	5	14	93.33	6.67	Fungal
	10	13	86.66	13.34	Fungal
	15	11	73.33	26.67	Fungal

Dm: Duration (min.); CE: Contaminated explants out of 15;  
C: Contamination (%); SA: Sterilization achieved (%);  
CT: Contamination type

### Genotypic response to shoot induction in nodal explants

To investigate genotype-specific responses in shoot induction, nodal explants were collected from three custard apple cultivars: Balanagar, Hybrid-12, and Phule Janaki. Given the low shoot induction efficiency in cv. Balanagar observed in initial trials, alternative genotypes were tested to assess their morphogenetic potential under identical conditions. The explants were cultured on MS medium containing varying concentrations of BAP and KIN: 0.4, 0.5, 0.6, 0.7, and 0.8 mg/L for both cytokinins (Table 2). The medium also contained 0.1 g/L activated charcoal. Cultures were incubated in darkness for the first week to enhance bud induction, followed by transfer to a 16/8-hour light/dark cycle for shoot development.

### Genotypic response to shoot induction in shoot tip explants

Shoot induction studies were also conducted using shoot tip explants from Balanagar and Hybrid-12 cultivars. Based on the protocol of Hepaksoy and Aksoy (2008), MS medium was supplemented with different concentrations of BAP and GA<sub>3</sub>. The hormonal combinations tested included BAP (2.0 mg/L) + GA<sub>3</sub> (0.5 mg/L) and BAP (4.0 mg/L) + GA<sub>3</sub> (0.5 mg/L) (Table 2). These treatments aimed to optimize direct shoot regeneration from apical tissues. For root induction, well-developed shoots were transferred to rooting media supplemented with IBA 50 – 100 mg/L.

## Results and Discussion

### Sterilization of explants

Aseptic conditions are essential for successful *in vitro* tissue culture. These conditions are influenced by several factors, such as the microenvironment of the donor plant, the sterilization techniques employed, and the cleanliness of the laboratory environment. Given the potential contamination of custard apple explants from the orchard, surface sterilization was employed. This

**Table 4:** Results of surface sterilization of shoot tip explants (cv. Balanagar).

Sterilant	Dm	CE	C	SA	CT
HgCl <sub>2</sub> (0.1%, w/v)	5	2	13.33	86.67	Fungal
	10	Browning	-	-	-
	15	Browning	-	-	-
NaOCl (2%, w/v)	5	11	73.33	26.67	Fungal
	10	9	60.00	40.00	Fungal
	15	8	53.33	46.67	Fungal

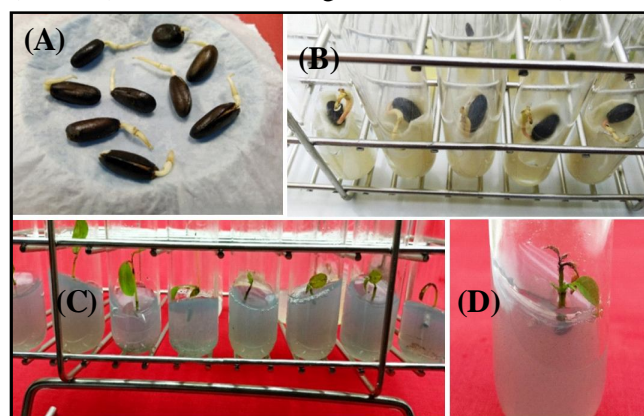
Dm: Duration (min.); CE: Contaminated explants out of 15;  
C: Contamination (%); SA: Sterilization achieved (%);  
CT: Contamination type

method depends on the concentration of the sterilizing agent and the duration of exposure. Over exposure to sterilants can increase explant mortality, whereas insufficient sterilization can lead to microbial contamination. Therefore, a careful balance must be maintained to preserve explant viability, ensuring that explants remain capable of differentiation and regeneration.

### Preliminary surface sterilization of cv. Balanagar explants

In the initial experiments, surface sterilization protocols were optimized for nodal and shoot tip explants of the cv. Balanagar, sourced from the AICRP on Arid Zone Fruits, M.P.K.V., Rahuri. The treatments included varying concentrations of HgCl<sub>2</sub> (0.1%, w/v) and NaClO (2%, w/v) for durations of 5, 10, and 15 minutes, followed by rinsing with 70% ethanol and sterile water. The results are shown in Tables 3 and 4.

The highest sterilization efficiency for nodal explants (93.33%) was achieved with HgCl<sub>2</sub> (0.1%, w/v) for 10 minutes, followed by 5 minutes of the same treatment (73.33%). In contrast, the highest contamination (93.33%)



**Fig. 2:** Seedling germination and shoot tip induction from shoot tip explants A: Germinated seeds. B: *In vitro* raised seedlings. C: Inoculation of shoot tips excised from seedlings germinated *in vitro*: D: Shoot initiated from a node of a seedling explant.

**Table 5:** Genotype response to surface sterilization treatment with HgCl<sub>2</sub> (0.1%, w/v) in nodal explants.

S.No.	Cultivar	CE	C	SA	CT
<b>For 5 min.</b>					
1	Balanagar	4	26.67	73.33	Fungal
2	Hybrid-12	6	40.00	60.00	Fungal
3	Phule Janaki	2	13.34	86.66	Fungal
<b>For 10 min.</b>					
1	Balanagar	1	6.67	93.33	Fungal
2	Hybrid-12	3	20.00	80.00	Fungal
3	Phule Janaki	Browning	-	-	-
<b>For 15 min.</b>					
1	Balanagar	Browning	-	-	-
2	Hybrid-12	1	6.67	93.33	Fungal
3	Phule Janaki	Browning	-	-	-
CE: Contaminated explants out of 15; C: Contamination (%); SA: Sterilization achieved (%); CT: Contamination type					

occurred with NaClO (2%, w/v) for 5 minutes. Longer HgCl<sub>2</sub> treatments (15 min) led to browning and death of the explants, indicating toxicity. For shoot tip explants, HgCl<sub>2</sub> (0.1%, w/v) for 5 minutes resulted in the greatest degree of sterilization (86.67%), whereas NaClO (2%, w/v) for 5 minutes led to the greatest degree of contamination (73.33%). Browning was observed in shoot tips treated with HgCl<sub>2</sub> for 10 or 15 minutes. Only fungal contamination was detected in the nodal and shoot tip explants. The impact of surface sterilization on the *in vitro* culture of explants is depicted in Fig. 1.

Establishing aseptic cultures of custard apple is challenging because of deep-seated endophytic contamination. Contaminated explants fail to survive long enough for growth regulators to elicit a response. This phenomenon, along with the browning of explants and culture media, is commonly encountered in tree tissue culture (Panchal and Patel, 2016). While surface sterilants can eliminate external microorganisms (Bonga, 1982), NaOCl was ineffective at controlling contamination in both nodal and shoot tip explants. Consequently, HgCl<sub>2</sub> proved to be more effective, as reported by Panchal and Patel (2016).

#### Genotypic response to HgCl<sub>2</sub> sterilization

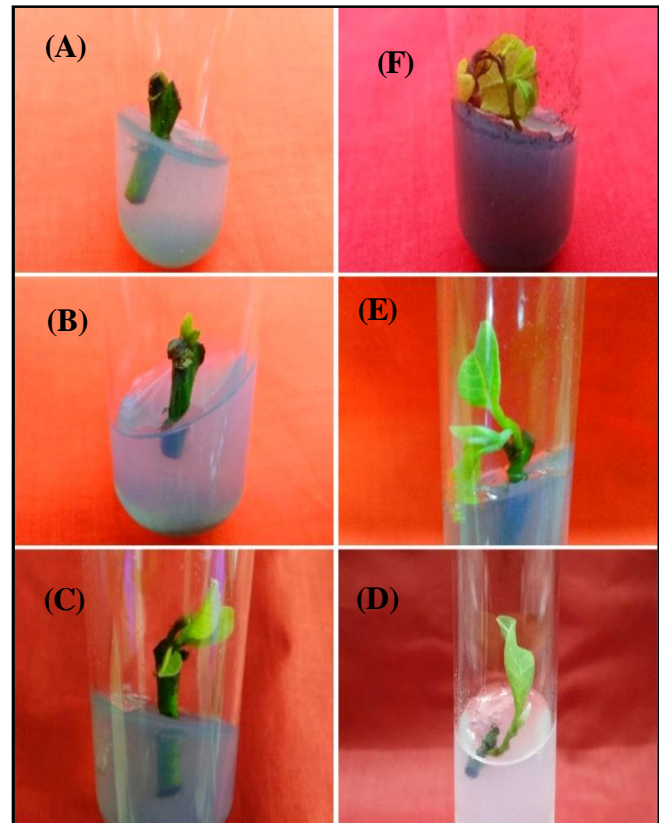
Three optimized HgCl<sub>2</sub> treatments (0.1%, w/v for 5, 10, and 15 minutes) were selected to compare surface sterilization across three custard apple cultivars: Balanagar, Hybrid-12, and Phule Janaki (Tables 5 and 6). For nodal explants, Balanagar had the highest disinfection rate (93.33%) with HgCl<sub>2</sub> (0.1%, w/v) for 10 minutes. Similarly, hybrid-12 achieved 93.33% sterilization with HgCl<sub>2</sub> for 15 minutes. Phule Janaki exhibited 86.66% sterilization with HgCl<sub>2</sub> for 5 minutes,

**Table 6:** Genotype response of shoot tip explants to surface sterilization treatment with 0.1% HgCl<sub>2</sub> (w/v).

S.No.	Cultivar	CE	C	SA	CT
<b>For 5 min.</b>					
1	Balanagar	0	0	100.00	Fungal
2	Hybrid-12	3	20.00	80.00	Fungal
3	Phule Janaki	2	13.34	86.66	Fungal
CE: Contaminated explants out of 15; C: Contamination (%); SA: Sterilization achieved (%); CT: Contamination type					

but prolonged treatment resulted in browning and death. Farooq *et al.*, (2002) also successfully sterilized nodal explants of custard apple cv. Balanagar using 0.1% HgCl<sub>2</sub> for 15 minutes. For shoot tip explants, Balanagar achieved 100% disinfection with HgCl<sub>2</sub> (0.1%, w/v) for 5 minutes, whereas Phule Janaki and Hybrid-12 showed high sterilization levels of 86.67% and 80%, respectively.

The response to surface sterilization varied between shoot tip explants and nodal explants, with nodal explants requiring longer exposure times. This is likely due to a greater degree of contamination in mature nodal explants, which may harbor more endogenous microbes (Dineshbabu *et al.*, 2002). As the exposure time and



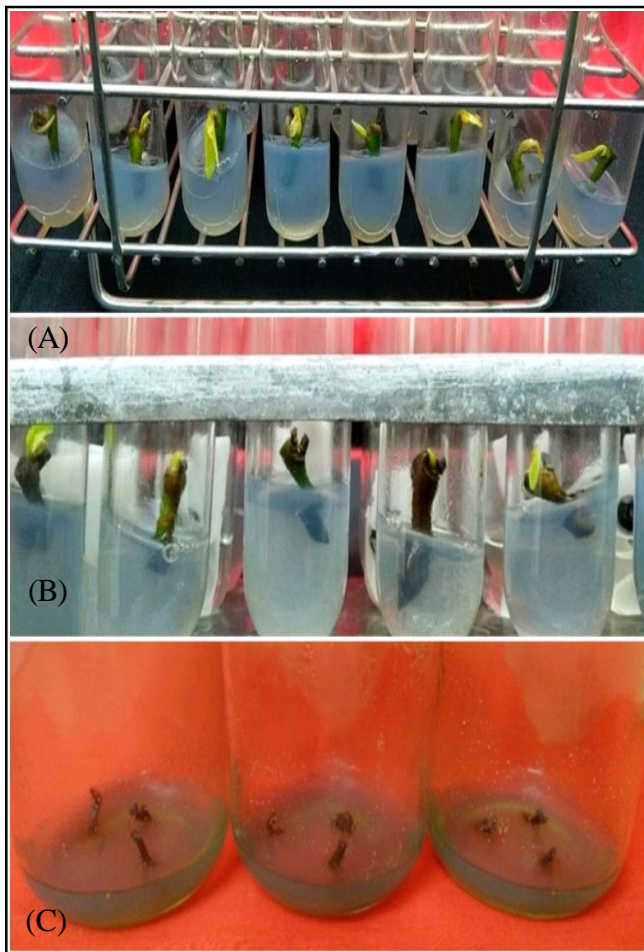
**Fig. 3:** Response to shooting media given by nodal explants A: Inoculated nodal explant; B: Shoot initiated from a nodal explant; C: Shoot development; D, E: 1<sup>st</sup> subculture of the nodal explants; F: 2<sup>nd</sup> subculture of shoots developed from nodal explants.

HgCl<sub>2</sub> concentration increased, contamination decreased, but prolonged treatment caused browning and eventual explant death.

Different genotypes respond differently to sterilization, likely due to variations in vascular tissue infection levels. Compared with nodal explants, shoot tip explants presented higher disinfection rates at shorter exposure times. Phule Janaki was more sensitive to prolonged HgCl<sub>2</sub> exposure, resulting in failure to establish on MS media with BAP and KIN. Among the cultivars, Hybrid-12 showed the greatest tolerance to HgCl<sub>2</sub>.

### Shoot induction

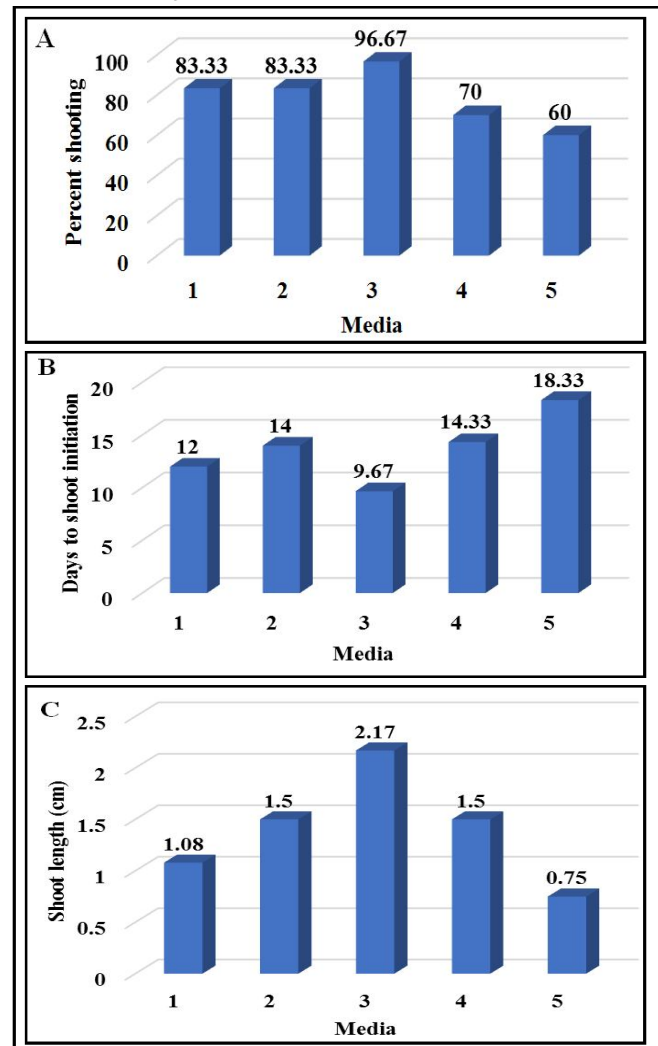
The axillary buds in custard apple are cryptic, hidden at the base of the petiole, and are released in two ways: after leaf abscission and during early shoot development among the first few nodes with subtending leaves still attached (Olesen and Muldoon, 2009). Flowering is limited to the early stages of shoot development and is primarily extra-axillary, occurring opposite the leaves



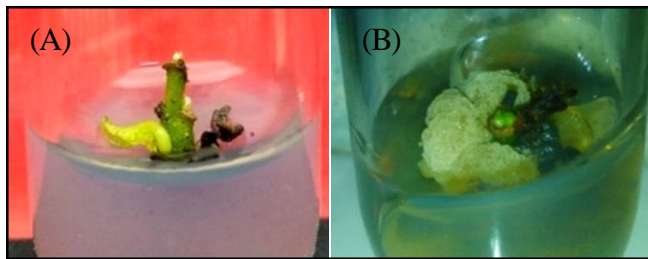
**Fig. 4:** Response of genotypes to shoot induction media for nodal explants A: Response of cv. Balanagar to shoot initiation media. B: Response of cv. Hybrid-12 to shoot initiation media. C: Response of cv. Phule Janaki to shoot initiation media.

(Venkataratnam, 1959; Thakur and Singh, 1965; George and Nissen, 1991). This flowering behavior necessitates rapid lateral bud development, highlighting the unique growth characteristics of the species.

In the present study, nodal segments and shoot tips were used, with a focus on shoot tip culture, axillary bud culture, and indirect organogenesis. Direct shoot regeneration was observed from nodal explants on MS media, whereas some shoot tip explants exhibited hampered regeneration due to callus formation. Shoots were successfully induced on MS basal media supplemented with various concentrations of BAP, KIN, IBA, and GA<sub>3</sub>.



**Fig. 5:** Effects of various media on shoot induction 1) MS + BAP (0.4 mg/l) + KIN (0.4 mg/l), 2) MS + BAP (0.5 mg/l) + KIN (0.5 mg/l), 3) MS + BAP (0.6 mg/l) + KIN (0.6 mg/l), 4) MS + BAP (0.7 mg/l) + KIN (0.7 mg/l), and 5) MS + BAP (0.8 mg/l) + KIN (0.8 mg/l) A: Effects of various media on the percentage of nodal explants. B: Effects of various media on the number of days to shoot initiation for nodal explants. C: Effects of various media on shoot length (cm) in nodal explants.

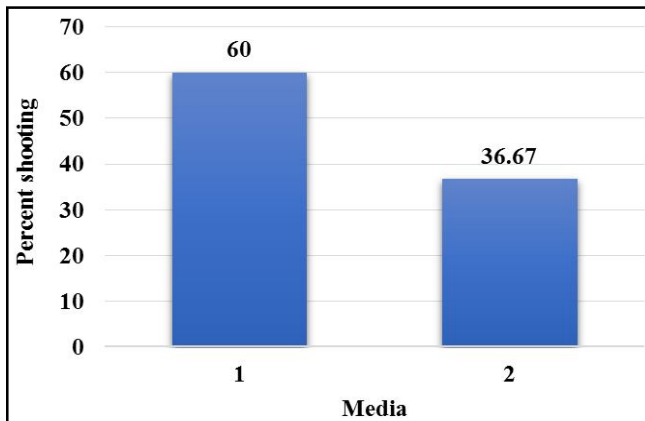


**Fig. 6:** Response of shoot tip explants to shooting media A: Shoot initiation from shoot tip explants. B: Callus growth at the base of shoot tip explants.

### Preliminary seedling germination and shoot tip induction under sterile conditions

To increase seed germination by overcoming dormancy, several pretreatments were applied, including mechanical scarification (seed coat removal), hot water treatment, and GA<sub>3</sub> hormone pretreatment (8.67 μM), following the methods suggested by Kudikala *et al.*, (2018). Among these treatments, hot water treatment resulted in the best response, in which intact seeds were exposed to 100°C water for 5 minutes, followed by immediate transfer to room temperature water. The seeds were then sterilized with 0.1% HgCl<sub>2</sub> for 5 minutes and rinsed with sterile distilled water 2–3 times. After the samples were soaked in sterile water for 24 hours, they were inoculated on paper moistened with distilled water. However, attempts to germinate seeds on paper bridges placed over liquid MS media were unsuccessful.

Seed germination occurred 9 to 10 days after inoculation, and shoots emerged 15 to 16 days after germination. One-week-old shoots were then excised and inoculated onto MS media supplemented with BAP and KIN. Leaf formation from the nodes of the seedling explants occurred 6 to 7 days later, although contamination due to endogenous microbes was observed. The process of seedling germination and shoot tip induction is depicted in Fig. 2.



**Fig. 7:** Effects of various media on the percentage of shoot tip explants 1) MS + BAP (2.0 mg/L) + GA<sub>3</sub> (0.5 mg/L) and 2) MS + BAP (4.0 mg/L) + GA<sub>3</sub> (0.5 mg/L).

**Table 7:** ANOVA showing the effects of genotype, media combination and their interaction on percent shoot induction, days to shoot initiation and shoot length (cm) for nodal explants.

Source (Treatments)	df	Mean Squares		
		PSI	DSI	SL
Genotype	1	577.23*	3.90**	0.65**
Media	4	1072.13**	0.98**	0.16**
Genotype × Media	4	101.57	0.20*	0.023*
Error	20	86.61	0.06	0.008

\*, \*\* Significant at the 5% and 1% levels, respectively  
PSI: Percent shoot induction; SL: Shoot length;  
DSI: Days to shoot initiation

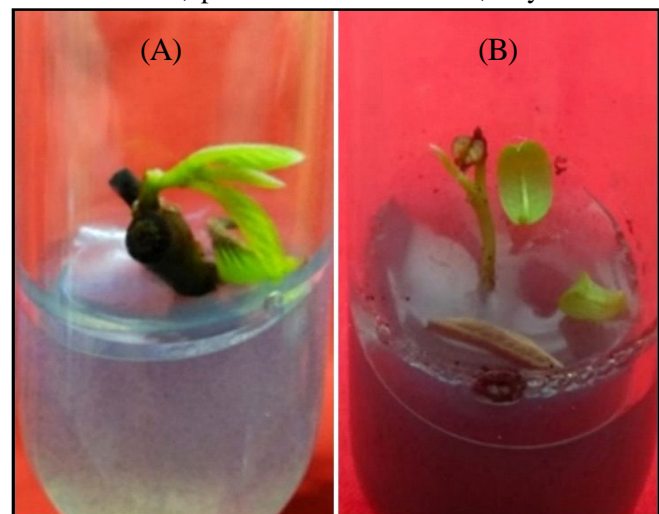
### Preliminary shoot tip induction in cv. balanagar using nodal explants

Except for the medium containing BAP (0.6 mg/L) + KIN (0.6 mg/L), all the media resulted in poor sprouting of the apical buds, which occurred in only rare instances. Even when sprouting was observed, it occurred late, after 4–5 weeks of culture and did not progress beyond the emergence of a single leaf. The response of the nodal explants to the shooting media is shown in Fig. 3.

### Genotypic response to shoot induction in nodal explants

Owing to the poor shoot induction response of cv. Balanagar to the previously tested media, two additional custard apple cultivars, Hybrid-12 and Phule Janaki, were included to investigate whether the responses were genotype specific. Phule Janaki was highly sensitive to HgCl<sub>2</sub> and did not survive, so it was excluded from further statistical analysis.

The analysis of variance (Table 7) revealed that the mean squares for the media were highly significant for all three traits, percent shoot induction, days to shoot



**Fig. 8:** Leaf abscission A: Leaf abscission at early stage. B: Leaf abscission after transfer to rooting media.

**Table 8:** ANOVA showing the effects of genotype, media combination and their interaction on percent shoot induction in shoot tip explants.

Source (Treatments)	df	Mean Squares for percent shoot induction
Genotype	1	1243.32**
Media	1	817.96**
Genotype × Media	1	0.004
Error	8	22.18

\*, \*\* Significant at the 5% and 1% levels, respectively

initiation, and shoot length indicating significant differences among the MS media with varying concentrations of BAP and KIN for these traits.

The genotype effect was highly significant for two traits, days to shoot initiation and shoot length, and significant for percent shoot induction. The genotype × media interaction was significant for days to shoot initiation and shoot length but not for percent shoot induction. The response of the genotypes to the shoot induction media is depicted in Fig. 4.

#### Percent shooting in nodal explants

The analysis of the mean and interaction effects for percent shoot initiation (Fig. 5a) revealed that the Balanagar genotype had a significantly greater shoot initiation rate (82.67%) than did the Hybrid-12 genotype (74.67%). Among the hormonal media tested, MS media supplemented with BAP (0.6 mg/l) + KIN (0.6 mg/l) led to the highest percentage of shoots (96.67%), whereas the lowest percentage (60.00%) was recorded with BAP (0.8 mg/l) + KIN (0.8 mg/l). Farooq *et al.* (2002) reported that equimolar concentrations of BAP and KIN were essential for efficient shoot induction, whereas Patnaik *et al.*, (2016) reported the success of BAP and NAA in *Annona squamosa* regeneration.

#### Days to shoot initiation in nodal explants

Comparisons of the mean and interaction effects for days to shoot initiation (Fig. 5b) revealed that Balanagar initiated shoots significantly faster (10.80 days) than did Hybrid-12 (16.53 days). The MS medium with BAP (0.6 mg/L) + KIN (0.6 mg/L) resulted in the shortest time to

shoot initiation (9.67 days), whereas the longest time (18.33 days) was observed with BAP (0.8 mg/L) + KIN (0.8 mg/L). The genotype × media interaction was significant, with Balanagar showing the fastest initiation (8.67 days) on BAP (0.6 mg/L) + KIN (0.6 mg/L), whereas Hybrid-12 required the most time (23.00 days) on BAP (0.8 mg/L) + KIN (0.8 mg/L). Farooq *et al.*, (2002) reported a shoot bud formation window of 4 to 14 days.

#### Shoot length in nodal explants

The analysis of shoot length (Figure 5c) revealed that, compared with Hybrid-12 (0.93 cm), Balanagar achieved the greatest shoot length (1.87 cm). Among the media, BAP (0.6 mg/L) + KIN (0.6 mg/L) produced the longest shoots (2.17 cm), whereas BAP (0.8 mg/L) + KIN (0.8 mg/L) resulted in the shortest shoots (0.75 cm). The genotype × media interaction revealed that Balanagar achieved the maximum shoot length (3.00 cm) on BAP (0.6 mg/L) + KIN (0.6 mg/L), whereas Hybrid-12 produced the shortest shoot length (0.50 cm) on BAP (0.8 mg/L) + KIN (0.8 mg/L). Farooq *et al.*, (2002) also reported shoot lengths of 2 to 4 cm.

#### Genotype response to shoot induction studies for shoot tip explants

The analysis of variance (Table 8) revealed that both genotype and media were highly significant factors for percent shoot induction, indicating distinct differences between genotypes and media for this trait. However, the interaction between genotype and media was not significant, suggesting that there was no genotype-specific response to the media treatments for shoot induction. The results are shown in Fig. 6.

#### Percent shooting in shoot tip explants

Among the two genotypes tested, Balanagar produced a significantly greater percentage of shoot induction (66.67%) than did Hybrid-12 (30.00%). Among the two hormonal media used, MS media containing BAP (2.0 mg/l) + GA<sub>3</sub> (0.5 mg/l) resulted in the highest percentage of shoots (60.00%), whereas the lowest percentage of shoots (36.67%) was observed on MS media

**Table 9:** The response to shoot establishment and callus interference (shoot tip explants).

Concentration and combination of hormones	Number of explants inoculated	Number of explants forming shoots	Number of explants forming callus	Percentage of response for shoot induction	Percentage of callus interference
<b>Balanagar</b>					
MS + BAP(2.0 mg/l) + GA <sub>3</sub> (0.5 mg/l)	15	12	3	80.00	20.00
MS + BAP(4.0 mg/l) + GA <sub>3</sub> (0.5 mg/l)	15	8	7	53.33	46.67
<b>Hybrid-12</b>					
MS + BAP(2.0 mg/l) + GA <sub>3</sub> (0.5 mg/l)	15	7	8	46.67	53.33
MS + BAP(4.0 mg/l) + GA <sub>3</sub> (0.5 mg/l)	15	3	12	20.00	80.00

supplemented with BAP (4.0 mg/l) + GA<sub>3</sub> (0.5 mg/l) (Fig. 7). Callus formation occurred at the base of explants across all the treatments, with the highest callus percentage observed in Hybrid-12 (80%) on BAP (4.0 mg/l) + GA<sub>3</sub> (0.5 mg/l). In contrast, Balanagar had lower callus formation (20%) in response to BAP (2.0 mg/L) + GA<sub>3</sub> (0.5 mg/L) (Table 9). The formation of calli, which begins 25 days after inoculation, interferes with shoot development. Freitas *et al.*, (2016) reported similar findings in *Annona emarginata* during *in vitro* culture, where high BAP concentrations led to the formation of “clumps” at the base of explants. These clumps often result in malformed leaves or buds, which are undesirable for clonal propagation due to potential somaclonal variation.

### Response to Rooting Media

After two successive subcultures of shoots on MS media with the same hormonal concentrations used for initiation (at 15-day intervals), shoots measuring 2–3 cm were transferred to rooting media containing various concentrations of auxin (IBA 50–100 mg/l), along with other components such as 2% sucrose, agar, and 1 g/L activated charcoal. A significant issue encountered during rooting is leaf abscission, which hinders root development. The explants shed their leaves before becoming sufficiently hardy or lignified for root initiation, ultimately leading to explant death. This issue is illustrated in Fig. 8. To address leaf abscission, silver nitrate (5 mg/l), as recommended by Panchal and Patel (2017), was added to the media. However, this treatment was only partially effective, as it merely delayed the onset of leaf shedding without preventing it. Transferring the shoots to rooting media containing silver nitrate failed to stop further leaf abscission.

According to Lemos and Blake (1994), *A. squamosa* exhibits a physiological mechanism for shedding leaves during drought conditions. The stress induced by harvesting twigs, sterilization, and culture in a controlled environment may lead to ethylene production, which can trigger physiological responses such as leaf abscission. Even low levels of ethylene produced by *Annona squamosa* explants in culture (0.1 µl/l/day) were sufficient to cause leaf shedding, complicating plantlet growth. Abeles *et al.*, (1992) noted that cultured microcuttings of *Annona squamosa* tend to release ethylene under stress, contributing to leaf abscission. Similarly, Farooq *et al.*, (2002) reported leaf abscission in *Annona squamosa* cultured on media supplemented with IBA. Freitas *et al.*, (2016) reported difficulty in stimulating root development during *in vitro* culture of *Annona emarginata*, regardless of the type, concentration, or duration of auxin treatment.

### Conclusion

The *in vitro* culture response of custard apple (*A. squamosa*) was highly genotype specific. Balanagar and Hybrid-12 showed positive responses, whereas Phule Janaki was highly sensitive to sterilization and *in vitro* conditions. Balanagar exhibited superior shooting from nodal and shoot tip explants, while Hybrid-12 showed callus induction. HgCl<sub>2</sub> (0.1%, w/v) for 10 min (nodal) and 5 min (shoot tip) was optimal for surface sterilization. MS medium supplemented with BAP (0.6 mg/L) + KIN (0.6 mg/L) was most effective for nodal shoot regeneration, while BAP (2.0 mg/L) + GA<sub>3</sub> (0.5 mg/L) was suitable for shoot tip explants. Rooting was unsuccessful due to severe leaf abscission. These findings provide a basis for refining micropropagation and developing transformation protocols in custard apple.

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### Abbreviations

MS: Murashige and Skoog, cv: Cultivar, HgCl<sub>2</sub>: Mercuric Chloride, NaClO: Sodium Hypochlorite, BAP: 6-Benzylaminopurine, KIN: Kinetin, GA<sub>3</sub>: Gibberellic Acid, IBA: Indole-3-Butyric Acid, mg/L: Milligram per Liter, w/v: Weight per Volume, µl/L/day: microliter per liter per day

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