



Direct shoot regeneration and establishment of *Solanum pseudocapsicum* from nodal and leaf segments

RAHMYA SHEFRIN¹, SUSHMITHA L C¹, SUSHMA SAGAR¹, NIKITA BALIYAN¹,
ARPITA SRIVASTAVA¹, ANIL KHAR² and MANISHA MANGAL^{1*}

ICAR-Indian Agricultural Research Institute, New Delhi 110 012, India

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ABSTRACT

The study was carried out during 2022 and 2023 at ICAR- Indian Agricultural Research Institute, New Delhi to establish a reliable and efficient protocol for direct regeneration of *Solanum pseudocapsicum* (L.) using nodal and leaf explants. Murashige and Skoog (MS) medium fortified with 0.5 mg/L kinetin and 0.2 mg/L indole-3-acetic acid (IAA) was found best for shoot regeneration, resulting in an average of 5.4 shoots/explant with an average shoot length of approximately 6.8 cm. Among the cytokinins tested, kinetin demonstrated superior efficacy compared to benzylaminopurine (BAP), while IAA outperformed α -naphthaleneacetic acid (NAA) among the auxins. The incorporation of gibberellic acid (GA₃) to kinetin-supplemented media further enhanced shoot induction and elongation. MS medium fortified with 0.5 mg/L GA₃ and kinetin at concentrations ranging from 1–3 mg/L resulted in 5.4–5.8 shoots per explant, with average increase in shoot lengths varying from 5.2–5.8 cm. Notably, no significant statistical differences were observed among the three kinetin concentrations tested (1, 2 and 3 mg/L), indicating that GA₃ was the critical factor for shoot elongation at this stage. Direct shoot regeneration was recorded from the leaf explants with the leaf margins showing pronounced growth within two weeks of inoculation which gave rise to shoots after one month of culture. The maximum number of shoots per leaf explant was achieved on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L NAA. Regenerated shoots were successfully rooted on MS basal medium.

Keywords: Direct organogenesis, *In vitro* propagation, Leaf curl virus, Solanaceae, *Solanum pseudocapsicum* L, Shoot regeneration

Solanum pseudocapsicum (L), a perennial shrub with a lifespan exceeding 10 years, shares morphological similarities with the genus *Capsicum*. Its unripe fruits possess antioxidant, antifungal, cytotoxic, and anti-tumor properties (Vijayan *et al.* 2008). Plant extracts exhibit strong insecticidal effects, inhibiting growth in cotton bollworms and armyworms (Jeyasankar *et al.* 2021). Recent studies have shown its strong resistance to chilli leaf curl disease (Srivastava *et al.* 2021), a major begomovirus affecting chillies in the Indian subcontinent (Kumar *et al.* 2015). *S. pseudocapsicum* also resists Pepino Mosaic virus (Aleixandre *et al.* 2007), powdery mildew (Bubici and Cirulli 2008), mites (Schalk *et al.* 1975), and verticillium wilt (Bletsos and Olympos 2008).

Solanum pseudocapsicum manifests a multifaceted resistance to various diseases. The incorporation of this resilient genetic resource into chili (*Capsicum annuum* L.) improvement programmes, however, presents a formidable

challenge due to its taxonomic distinction, giving rise to potential fertilization barriers arising from disparate genera. To address this challenge the strategic application of advanced *in vitro* culture methodologies, such as embryo rescue and *in vitro* fertilization, appear to be viable alternatives. Therefore, *Solanum pseudocapsicum* has become an important candidate as a resistance donor for chili improvement programs, enabling the introgression of chili leaf curl virus (ChiLCV) resistance into cultivated *Capsicum annuum* by transferring specific resistance genes from the former into the latter through hybridization and backcrossing, while retaining desirable traits of cultivated varieties. The utilization of such biotechnological tools necessitates the standardization of an efficient protocol for *in vitro* regeneration of *S. pseudocapsicum*, however there has been limited exploration of this species for *in vitro* propagation.

In our study, we standardized a protocol for direct organogenesis in *Solanum pseudocapsicum*, utilizing nodal segments excised from plants grown in field and leaf explants derived from *in vitro* regenerated shoots. This effort aims to facilitate in-depth research and explore potential breeding approaches for *S. pseudocapsicum* in the future.

¹ICAR-Indian Agricultural Research Institute, New Delhi;

²ICAR-Indian Agricultural Research Institute, Regional Station, Pune, Maharashtra. *Corresponding author email: manishamangal@rediffmail.com

MATERIALS AND METHODS

The study was carried out during 2022 and 2023 at ICAR-Indian Agricultural Research Institute, New Delhi. The young healthy shoots with nodes, specifically with the axillary buds and fully expanded leaves were utilized as the explants for *in vitro* regeneration (Fig. 1A and 1B). 10 explants per flask were used for shoot induction. Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962), fortified with varying concentrations and combinations of cytokinins, auxins, and GA₃ was utilized to investigate shoot and root induction in *Solanum pseudocapsicum* (Tables 1, 2, 3, and Supplementary Table 1). The type and concentrations of different auxins and cytokinins were used as per literature study.

For the sterilization of the explants, nodal explants were initially rinsed in tap water, followed by treatment with a solution of 2–3 drops of Tween 20/100 ml and Bavistin for 10–12 min. The explants were rinsed multiple times with tap water until all traces of foam, Tween 20, and Bavistin (0.2% w/v) were removed. In a laminar airflow hood, explants were rinsed with autoclaved distilled water, sterilized with 0.5% HgCl₂ for 4 min, rinsed again, then treated with 70% ethanol for 1 min, and rinsed thrice with autoclaved distilled water afterwards to remove any residual sterilants.

Surface-sterilized explants were trimmed to a single node, approximately 1–1.5 cm in length, before inoculation. While, fully developed sterile leaves, excised from regenerated nodal explants, were trimmed to an area of ~1 cm² and inoculated onto MS medium supplemented with varying concentrations of BAP, and NAA. Table 1 shows the media employed for initial shoot induction whereas, effect of callus induction media on leaf explants from *in vitro* established shoots of *S. pseudocapsicum* is shown in Table 2. Table 3 shows the media where initial shoots were sub-cultured to get shoot elongation while Supplementary Table 1 illustrates the response of shoots on various root induction media.

Cultures were maintained under 50 µmol/m²/s fluorescent light with a 16/8 h photoperiod at 25 ± 2°C. One-month-old regenerated shoots were sub-cultured every 15 days onto fresh multiplication media (Table 3). Healthy leaves from regenerated shoots were used for inoculation, while regenerated shoots (~2.5–5 cm in length) were shifted to rooting media either MS basal medium or MS medium fortified with auxins (Supplementary Table 1).

Rooted shoots were transferred to autoclaved test tubes with 15–20 ml of Hoagland's solution (Hoagland and Synder 1933) for primary hardening and incubated for one week. Afterward, plantlets were moved to a growth chamber (22 ± 2°C, 60% humidity, 16/8 h photoperiod). For secondary hardening, well-rooted plantlets were shifted to plastic pots with a cocopeat-perlite-vermiculite mixture (2:1:1).

The experiment followed a completely randomized design (CRD), with each treatment comprising 10 explants and replicated thrice. Parameters recorded included per cent bud break, the number of nodes/shoot, shoot length, the number of shoots per explant, and the number of roots. Statistical study was performed using one-way analysis of variance (ANOVA) in R software.

RESULTS AND DISCUSSION

Morphogenesis was recorded for all the treatments, although the number of occurrences were lower in a few treatments (Table 1). Bud break was observed on the fourth day following inoculation (Fig. 1B) and within two weeks of inoculation, the explants regenerated leaves (Fig. 1C). MS media with the combinations of 1.0 mg/L BAP +

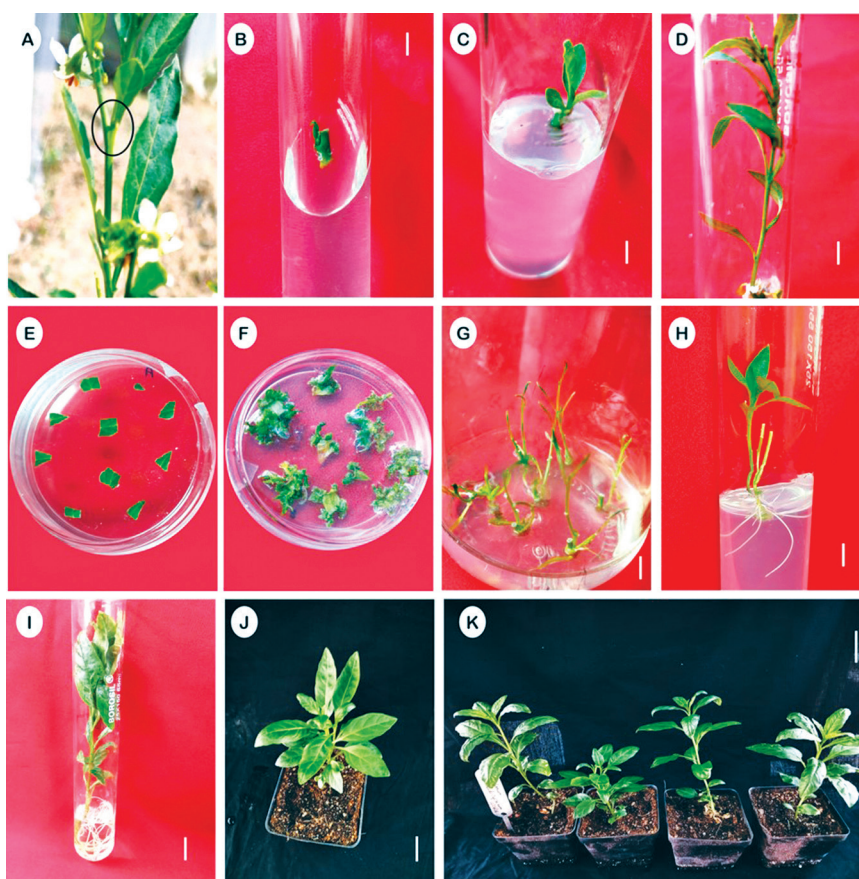


Fig. 1 (A) Nodal segment used as explant; (B) Bud break after the fourth day of inoculation: Scale bar = 0.5 cm; (C and D) Shoot regeneration after the third week of the culture: Scale bar = 1 cm; (E) Leaf explant from the regenerated shoots; (F) Shoot regeneration from the leaf explants; (G) Shoot multiplication and proliferation in the shoot multiplication medium: Scale bar = 1 cm; (H) Rooting of the shoots on MS media: Scale bar = 1 cm; (I) Primary hardening on Hoagland's solution: Scale bar = 2 cm; (J and K) Hardened healthy plants: Scale bar = 5 cm.

Table 1 Effect of different combinations of shoot induction media on nodal explants of *S. pseudocapsicum*

Experimental set	Media combination (mg/L)	Average increase in length of the shoots/explant	Average number of nodes/explant	Bud break percentage (%)
T ₁	0.5 BAP + 0.1 IAA	0.6 ± 0.89 ^e	0.8 ± 1.3 ^{cd}	40 ± 0.00 ^{de}
T ₂	1.0 BAP + 0.1 IAA	2.8 ± 0.84 ^{bc}	2.4 ± 1.34 ^b	100 ± 0.00 ^a
T ₃	1.5 BAP + 0.1 IAA	0.8 ± 1.3 ^{de}	0.4 ± 0.55 ^d	37 ± 4.47 ^{de}
T ₄	0.5 BAP + 0.2 IAA	0.4 ± 0.89 ^e	0.2 ± 0.45 ^d	16 ± 5.48 ^h
T ₅	1.0 BAP + 0.2 IAA	0.4 ± 0.89 ^e	0 ± 0 ^d	18 ± 4.47 ^h
T ₆	1.5 BAP + 0.2 IAA	0.4 ± 0.55 ^e	0 ± 0 ^d	38 ± 2.74 ^{de}
T ₇	0.5 BAP + 0.1 NAA	0.2 ± 0.45 ^e	0.2 ± 0.45 ^d	18 ± 4.47 ^h
T ₈	1.0 BAP + 0.1 NAA	0.2 ± 0.45 ^e	0.2 ± 0.45 ^d	24 ± 5.48 ^{fgh}
T ₉	1.5 BAP + 0.1 NAA	0.4 ± 0.89 ^e	0.2 ± 0.45 ^d	20 ± 7.07 ^h
T ₁₀	0.5 BAP + 0.2 NAA	0.2 ± 0.45 ^e	0.2 ± 0.45 ^d	22 ± 10.95 ^{gh}
T ₁₁	1.0 BAP + 0.2 NAA	0.2 ± 0.45 ^e	0.2 ± 0.45 ^d	24 ± 5.48 ^{fgh}
T ₁₂	1.5 BAP + 0.2 NAA	0.4 ± 0.55 ^e	0.2 ± 0.45 ^d	32 ± 8.37 ^{efg}
T ₁₃	0.5 KIN + 0.1 IAA	3.94 ± 0.99 ^b	2.6 ± 1.34 ^b	96 ± 8.94 ^a
T ₁₄	1.0 KIN + 0.1 IAA	1.9 ± 1.82 ^{cde}	2 ± 1.87 ^{bc}	52 ± 10.95 ^c
T ₁₅	1.5 KIN + 0.1 IAA	2.4 ± 1.52 ^{bcd}	2.4 ± 1.67 ^b	72 ± 10.95 ^b
T ₁₆	0.5 KIN + 0.2 IAA	6.8 ± 3.11 ^a	5.4 ± 1.95 ^a	100 ± 0 ^a
T ₁₇	1.0 KIN + 0.2 IAA	1 ± 1.41 ^{de}	0.6 ± 0.89 ^d	36 ± 5.48 ^{de}
T ₁₈	1.5 KIN + 0.2 IAA	0.6 ± 0.89 ^e	0.2 ± 0.45 ^d	36 ± 5.48 ^{de}
T ₁₉	0.5 KIN + 0.1 NAA	3.2 ± 2.29 ^{bc}	3 ± 1.87 ^b	90 ± 14.14 ^a
T ₂₀	1.0 KIN + 0.1 NAA	0.5 ± 0.71 ^e	0.2 ± 0.45 ^d	34 ± 8.94 ^{def}
T ₂₁	1.5 KIN + 0.1 NAA	0.4 ± 0.55 ^e	0.2 ± 0.45 ^d	38 ± 14.83 ^{de}
T ₂₂	0.5 KIN + 0.2 NAA	0.4 ± 0.55 ^e	0.2 ± 0.45 ^d	38 ± 4.47 ^{de}
T ₂₃	1.0 KIN + 0.2 NAA	0.4 ± 0.55 ^e	0.2 ± 0.45 ^d	44 ± 5.48 ^{cd}
T ₂₄	1.5 KIN + 0.2 NAA	0.5 ± 0.71 ^e	0.2 ± 0.45 ^d	42 ± 10.95 ^{cde}
	CV	97.25	104.9	17.46
	CD	1.38	0.94	9.74

Treatment details are given under Materials and Methods.

0.1 mg/L IAA (T₂) and 0.5 mg/L KIN + 0.2 mg/L IAA (T₁₆) demonstrated 100% bud break followed by 0.5 mg/L KIN + 0.1 mg/L IAA (T₁₃), and 0.5 mg/L KIN + 0.1 mg/L NAA (T₁₉) which also produced statistically similar results as shown in Table 1. The maximum number of nodes (more than 6 nodes) and the greatest shoot length (greater than 10 cm) were observed in the explants placed on MS media fortified with 0.5 mg/L KIN + 0.2 mg/L IAA (T₁₆ of Shoot regeneration media) within 4 weeks of inoculation (Table 1). T₁₆ was thus found optimum media combination for efficient shoot regeneration in *S. Pseudocapsicum*.

The trimmed leaves, measuring approximately 1 cm², excised from the established shoots were cultured for induction of callus on MS medium fortified with different combination of BAP and NAA (Table 2) (Fig. 1E). Unexpectedly, direct shoot regeneration was observed from the leaf explants bypassing the callus formation stage, after one month of culture (Fig. 1F). The maximum shoots per explant was achieved on MS medium fortified with 2.0 mg/L BAP and 0.5 mg/L NAA. These induced shoots were subsequently transferred to the shoot multiplication

Table 2 Effect of callus induction media on leaf explants from *in vitro* established shoots of *S. pseudocapsicum*

Experimental set	Media combination (mg/L)	Average number of shoots induced/explant
T ₁	2.0 BAP + 0.5 NAA	6.9 ± 0.37 ^a
T ₂	1.0 BAP + 1.0 NAA	5.7 ± 0.20 ^b
T ₃	1.0 BAP + 0.5 NAA	5.2 ± 0.40 ^b
T ₄	2.0 BAP + 1.0 2,4 - D	2.0 ± 1.00 ^c
T ₅	2.0 BAP + 0.5 2,4 - D	2.4 ± 0.75 ^c
	CD	1.12
	CV	13.91

Treatment details are given under Materials and Methods.

medium outlined in Table 3. The observed results deviated from those reported earlier in other studies. For instance, Sidik *et al.* (2020) obtained a high frequency of callus induction in leaf explants of *Solanum* spp. cultured on MS medium fortified with 2.0 mg/L BAP and 0.5 mg/L NAA. Similarly, Ray *et al.* (2011) demonstrated 48% callus induction in *Solanum melongena* cv. Jhumki under the same hormonal combination.

Table 3 Effect of different combinations of shoot multiplication media for *S. pseudocapsicum*

Experimental set	Media combination (mg/L)	Average length of the shoots/explant	Average number of shoots/explant
T ₁	1.0 KIN + 0.5 GA ₃	5.5 ± 1.0 ^a	5.6 ± 1.3 ^a
T ₂	2.0 KIN + 0.5 GA ₃	5.2 ± 0.90 ^a	5.4 ± 1.14 ^a
T ₃	3.0 KIN + 0.5 GA ₃	5.8 ± 1.60 ^a	5.8 ± 2.16 ^a
T ₄	1.0 BAP + 0.5 GA ₃	2.5 ± 2.39 ^b	1.6 ± 1.51 ^b
T ₅	2.0 BAP + 0.5 GA ₃	2 ± 2.12 ^b	0.8 ± 0.68 ^b
	CD	2.92	2.16
	CV	40.72	38.27

Treatment details are given under Materials and Methods.

Our study provides an optimized protocol for direct organogenesis of *S. pseudocapsicum* using nodal explants from field-grown plants. Previous research showed MS medium with BAP and IAA or NAA promotes direct organogenesis in solanaceous crops (Kumar *et al.* 2009, Nath and Das 2014). However, our results demonstrate that kinetin (KIN) outperforms BAP for both shoot induction and multiplication. The highest shoot induction efficiency was achieved on MS medium with 0.5 mg/L KIN and 0.2 mg/L IAA. For shoot proliferation and elongation, MS medium with 3.0 mg/L KIN and 0.5 mg/L GA₃ was found most effective.

The superiority of kinetin in promoting shoot organogenesis aligns with findings in related species. For instance, in a study on *Solanum nigrum*, researchers found that KIN was more effective than BAP in facilitating shoot multiplication as well as proliferation (Padmapriya *et al.* 2011). The literature study reveals that the effectiveness of cytokinins like 6-Benzylaminopurine (BAP) and kinetin for *in vitro* shoot induction varies according on the plant species and genotype. In many cases, BAP has been found more effective in promoting shoot multiplication. For instance, in lemongrass (*Cymbopogon citratus*), BAP at a concentration of 10 µM induced higher shoot proliferation compared to other cytokinins (Quiala *et al.* 2016). In potato (*Solanum tuberosum*), the response to cytokinins was found to be genotype-dependent, with BAP and Kinetin showing varying effectiveness across different genotypes (Muhammad *et al.* 2012). However, in cucumber (*Cucumis sativus*), kinetin was observed to be more effective for shoot proliferation (Abu-Romman *et al.* 2015). These findings suggest that the empirical testing is often necessary to determine the most effective cytokinin for a given plant species. Our results underscore kinetin's potential as a more efficient cytokinin for overcoming the inherent challenges of regeneration in *S. pseudocapsicum*, offering an enhanced protocol for *in vitro* culture in this and similar solanaceous species.

Our findings indicate that comparatively lower concentrations of cytokinins and auxins are sufficient for the induction, multiplication, and elongation phases of *in vitro* organogenesis in *S. pseudocapsicum*, aligning with observations from select previous studies (Nath and Das 2014, Kumar *et al.* 2011). The direct shoot regeneration in *S. pseudocapsicum* and other Solanaceae crops has traditionally utilized BAP in combination with the auxins or TDZ for the shoot induction (Nath and Das 2014, Arshlyonel *et al.* 2023). While our results are consistent with the previous reports in demonstrating the efficacy of cytokinin-auxin combinations for shoot induction, the optimized medium composition in our study (T₁₆, 0.5 mg/L KIN and 0.2 mg/L IAA) yielded the maximum shoot number and shoot length. The regenerated shoots were then sub-cultured on the media specified in Table 3 for shoot elongation and multiplication (Fig. 1G). The highest number of shoots (more than 9/explant) and maximum shoot length (greater than 8.0 cm) were observed on MS medium fortified with 3.0 mg/L KIN and 0.5 mg/L GA₃ (T₃). Lower concentrations of KIN (1.0 mg/L and 2.0 mg/L) in combination with GA₃ supported moderate shoot multiplication and elongation, producing an average of 5.6 and 5.4 shoots per explant with shoot lengths of 5.5 cm and 5.2 cm, respectively (T₁ and T₂). However, media containing BAP in combination with GA₃ (T₄ and T₅) yielded significantly lower shoot multiplication and elongation. These results establish that the MS medium fortified with 3.0 mg/L KIN and 0.5 mg/L GA₃ (T₃) is more suitable for enhanced shoot multiplication proliferation and elongation.

Explants under three weeks old showed better responses for shoot induction, highlighting the importance of using younger explants for successful *in vitro* organogenesis, as seen in previous studies (Santana-Buzzy *et al.* 2005). Elongated shoots were shifted to basal MS medium and MS medium with varying concentrations of IAA, IBA, and NAA (Supplementary Table 1) for rooting (Fig. 1H and 1I). Rooting was 100% successful within two weeks in both media. Basal MS medium promoted long, healthy primary and lateral roots, while auxin-supplemented media produced short, thick primary roots. Previous studies suggest roots have higher endogenous IAA levels than shoots (Trifunovic *et al.* 2016), with auxins synthesized in leaf tips and stored near roots (Aloni *et al.* 2003). Tomato explants formed adventitious roots without exogenous auxins due to endogenous auxin accumulation (Scarpella *et al.* 2006).

In our study, highest rooting efficiency with long and thin roots was achieved on basal MS medium, producing an average of 4.16 ± 0.37 roots per explant (T₁). MS fortified with IAA (0.1mg/L) (T₂) performed better (1.8 ± 0.44 roots/explants) than media supplemented with IBA or NAA. The rooted plants from the basal MS medium and MS fortified with IAA exhibited optimum elongation and induction of lateral roots (13.0 ± 0.62, 8.8 ± 1.15) and healthy leaves when placed in the Hoagland solution for primary hardening (Fig. 1J). However, during secondary

hardening in the growth chamber, some leaves turned yellow, a condition that resolved upon transferring the plantlets to the nursery (Fig. 1K). We observed a higher average number of roots per explant on MS basal medium (4.16 ± 0.37) exhibiting induction of long and healthy roots followed by MS supplemented with IAA (1.8 ± 0.44) (Supplementary Table 1). It was recorded that incorporation of rooting hormones (IAA, IBA, and NAA) resulted in comparatively lesser number of average roots per explant which were short and thick in appearance. Our results align with previous studies on Solanaceous crops. A 100% root induction was achieved in *Solanum tuberosum* (Abdelaleem 2015) using basal MS medium and MS with IBA. Similar findings in tomato by Devi *et al.* (2008) and Oguz *et al.* (2021) showed exogenous auxin was not essential for rooting.

Primary hardening is essential for transitioning tissue-cultured plantlets to *ex vitro* conditions. Hoagland's solution (Hoagland and Synder 1933), commonly used in hydroponics, has been applied in the hardening of plants like *Dioscorea floribunda*, Chrysanthemum, and date palm (Ben-Jaacov and Langhans 1972, Sita *et al.* 2015, Hassan 2017). It promoted better root area, length, vitality, chlorophyll content, and shoot growth in cucumber seedlings (Li and Cheng 2015). In our study on *Solanum pseudocapsicum*, Hoagland's solution in primary hardening enhanced root and shoot formation, with no morphological changes, ensuring 100% survival during secondary hardening.

In vitro propagation of wild perennial shrubs is challenging due to the difficulty in establishing contamination-free explants. The novelty of the study lies in its comprehensive development of an optimized *in vitro* regeneration protocol for *Solanum pseudocapsicum*, which addresses the challenges of contamination-free explant establishment and successful shoot multiplication. Our study demonstrates the superiority of KIN in conjunction with IAA and GA₃ over BAP for enhanced shoot induction, regeneration, and multiplication in *Solanum pseudocapsicum*. The best medium for shoot regeneration was identified as MS medium fortified with 0.5 mg/L KIN and 0.2 mg/L IAA. Further refinement led to successful shoot multiplication on MS medium fortified with 3.0 mg/L KIN and 0.5 mg/L GA₃. The established protocol not only facilitates *in vitro* organogenesis but also holds promise for large-scale production of *Solanum pseudocapsicum*. The *in vitro* propagation protocol developed for *Solanum pseudocapsicum*, can be adapted for other Solanaceae crops like *Capsicum annuum* and *Solanum lycopersicum* and their wild relatives by optimizing hormone concentrations for each species. Moreover, the protocol's integration with disease resistance traits, such as ChiLCV resistance, offers a pathway for accelerated breeding programs, facilitating the introgression of resistance genes into cultivated varieties through embryo rescue and genetic transformation.

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