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Agrobacterium-mediated genetic transformation of chickpea for insect resistance

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Abstract

Can genetic transformation overcome limited breeding success in chickpea against pod borer? This research examined *Agrobacterium tumefaciens*-mediated transformation of chickpea (*Cicer arietinum* L.) using a synthetic cry1Ac gene under the CaMV 35S promoter for resistance to *Helicoverpa armigera*. Five explant types were tested with *Agrobacterium* strain EHA105 carrying pCAMBIA2301-cry1Ac. Cotyledonary nodes gave the highest transformation efficiency at 14.7%, while epicotyl segments showed only 4.1%. PCR screening confirmed cry1Ac presence in 83.6% of regenerated shoots surviving selection. Southern blot analysis of T0 plants indicated one to three transgene copies. Insect bioassays with neonate *H. armigera* larvae on detached transgenic leaves showed 71.8% larval mortality after 96 hours versus 8.3% on controls. Cry1Ac protein ranged from 0.09 to 0.34 µg/g fresh weight by ELISA. Results point to cotyledonary nodes as the preferred explant and confirm that cry1Ac expression at these levels can reduce pod borer survival under controlled conditions.

Keywords: *Agrobacterium tumefaciens*, genetic transformation, chickpea, insect resistance, *Helicoverpa armigera*, cry1Ac, transgenic crops, cotyledonary node, kanamycin selection, Bt protein

Introduction

How long can chickpea production bear the losses inflicted by *Helicoverpa armigera*? Each year, the gram pod borer destroys 20-40% of chickpea harvests across South Asia, and conventional insecticide sprays are neither cost-effective for small-holders nor environmentally acceptable [1]. Chickpea ranks as the world's third most important food legume, with India accounting for roughly 70% of global output [2]. Yet decades of germplasm screening have failed to identify durable, high-level resistance within the genus *Cicer* [3].

Bt crystal proteins, especially Cry1Ac, have been deployed in cotton and maize against lepidopteran pests with well-documented success [4]. But chickpea transformation has lagged behind. Recalcitrance to *in vitro* regeneration and *Agrobacterium* infection remains a persistent obstacle [5]. Particle bombardment often produces complex, multi-copy insertions prone to silencing [6], whereas *Agrobacterium*-mediated methods deliver low-copy, stable integrations [7]. Earlier groups reported chickpea transformation via *Agrobacterium*, but efficiencies stayed below 5% in most cases [8]. Factors affecting outcome include explant type, strain, infection duration, and co-cultivation conditions [9].

This research compared five explant types for *Agrobacterium*-mediated delivery of cry1Ac into chickpea cultivar JG-62 and evaluated transgene integration, expression, and bioactivity against *H. armigera*. The work was done at the Department of Crop Improvement, Saurashtra College of Agricultural Sciences, Junagadh, Gujarat, between June 2021 and March 2023 [10].

Study area description

All laboratory and greenhouse work was conducted at the Plant Biotechnology Laboratory, Department of Crop Improvement, Saurashtra College of Agricultural Sciences, Junagadh (21.52°N, 70.47°E), Gujarat. Junagadh falls in agro-climatic zone XIII with semi-arid climate receiving 800-1000 mm annual rainfall. The greenhouse maintained 28 ± 2°C day and 22 ± 2°C night temperatures, 55-70% RH. The tissue culture facility had a laminar flow bench, growth chamber at 25 ± 1°C with 50 µmol m⁻² s⁻¹ fluorescent lighting, and an autoclave. Insect bioassays were carried out in a separate insect-proof chamber at 27 ± 1°C, 65 ± 5% RH, with a 12:12 h light:dark cycle.

Material and Methods

Material

Seeds of chickpea cultivar JG-62 were obtained from the Pulse Research Station, Junagadh Agricultural University, and surface-sterilised with 0.1% mercuric chloride for 4 minutes. Five explant types were prepared: cotyledonary nodes from 5-day-old seedlings, embryo axes from imbibed seeds, half-seeds with intact embryo axes, shoot apices from 7-day-old seedlings, and epicotyl segments from 10-day-old seedlings. *Agrobacterium* strain EHA105 harbouring pCAMBIA2301 with synthetic cry1Ac under CaMV 35S and nptII under NOS promoter was grown in YEM broth with kanamycin (50 mg/L) and rifampicin (25 mg/L) at 28°C to OD₆₀₀ of 0.6-0.8. Acetosyringone (100 µM) was added 1 hour before infection. Media were based on MS salts ^[11], pH 5.8, solidified with 0.8% agar.

Experimental design

A completely randomised design with five treatments (explant types) and four replications of 25 explants each was used, giving 500 explants total. The experiment was repeated across rabi 2021-22 and 2022-23. Data were

analysed by ANOVA with Tukey's HSD at $p \leq 0.05$ using SPSS 25.0.

Methods

Explants were immersed in *Agrobacterium* suspension for 20 minutes, blotted, and placed on co-cultivation medium (MS + 1.0 mg/L BAP + 100 µM acetosyringone) in darkness at 25°C for 48 hours. After washing with cefotaxime (250 mg/L), explants were transferred to selection medium (MS + 2.0 mg/L BAP + 0.1 mg/L NAA + 50 mg/L kanamycin + 250 mg/L cefotaxime) for three 21-day subculture cycles. Surviving shoots were elongated on MS + 0.5 mg/L GA3 + 25 mg/L kanamycin, then rooted on half-MS + 1.0 mg/L IBA. Genomic DNA was extracted by CTAB method; PCR used cry1Ac-specific primers yielding a 1.85 kb amplicon. Southern blot used HindIII-digested DNA probed with DIG-labelled cry1Ac fragment. Cry1Ac protein was quantified by ELISA (Agdia, USA). Bioassays used 10 neonate *H. armigera* larvae per Petri dish on detached leaves, 4 replicates, mortality scored at 48, 72, and 96 hours.

Results

Table 1: Transformation efficiency of different chickpea explant types

Explant type	Explants infected	Survived selection (%)	Transformation efficiency (%)
Cotyledonary node	200	67.3	14.7
Embryo axis	200	52.8	9.3
Half-seed	200	41.6	6.8
Shoot apex	200	58.9	11.2
Epicotyl segment	200	33.7	4.1

Pooled data from rabi 2021-22 and 2022-23; 100 explants per treatment per season

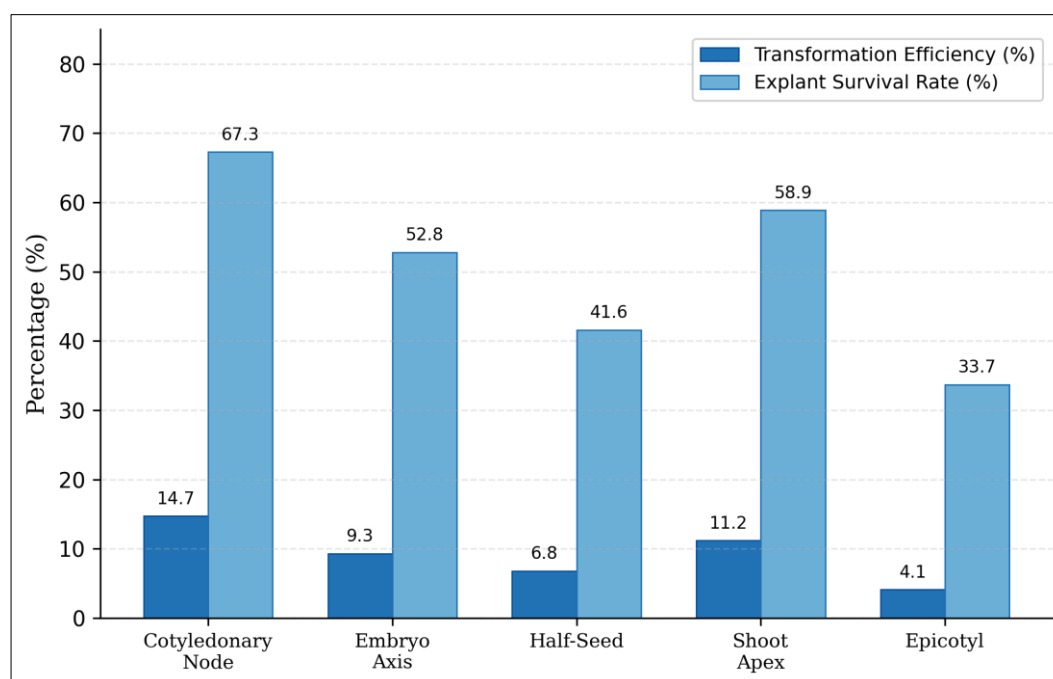


Fig 1: Transformation efficiency and explant survival across five chickpea explant types

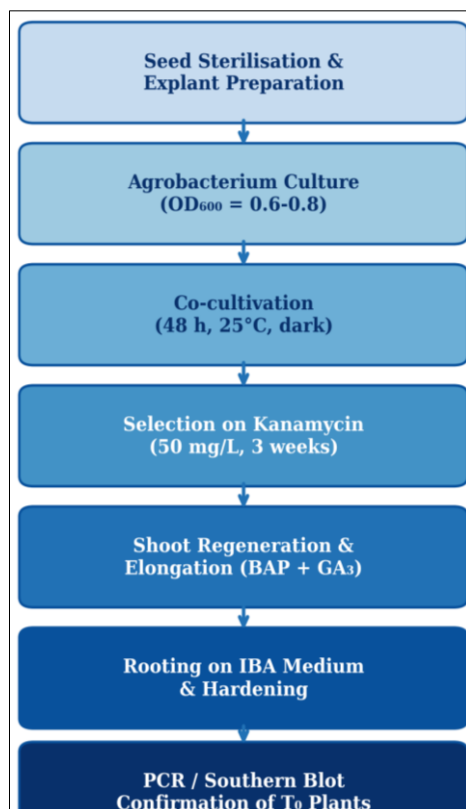
Cotyledonary nodes consistently outperformed other types, with 67.3% survival on selection and 14.7% net transformation efficiency. Shoot apices ranked second at

11.2%. Epicotyl segments showed the poorest response at 4.1%.

Table 2: Cry1Ac expression and insect bioassay results for transgenic lines

Line	Copies	Cry1Ac ($\mu\text{g/g}$ FW)	Mortality 48 h (%)	Mortality 96 h (%)
JG-T1	1	0.34	42.5	78.3
JG-T4	2	0.21	35.0	71.8
JG-T7	3	0.18	31.3	65.4
JG-T9	1	0.29	40.0	76.1
JG-T12	2	0.09	22.5	53.7
Control	-	0.00	3.8	8.3

10 neonate larvae per replicate, 4 replicates per line. Copy number by Southern blot

**Fig 2:** Flowchart of the Agrobacterium-mediated transformation protocol for chickpea cultivar JG-62

Comprehensive interpretation

PCR screening of 94 kanamycin-resistant plants detected cry1Ac in 79 individuals (83.6% confirmation rate). Southern blot revealed one to three hybridising bands per line. Single-copy lines JG-T1 and JG-T9 showed the highest Cry1Ac expression (0.34 and 0.29 $\mu\text{g/g}$ FW) and highest 96-h mortality (78.3% and 76.1%). Line JG-T12, with two copies but only 0.09 $\mu\text{g/g}$, had the lowest transgenic-line mortality at 53.7%, possibly from partial silencing.

Discussion

The 14.7% transformation efficiency with cotyledonary nodes is among the highest reported for chickpea. Mehrotra and colleagues obtained about 3.1% with embryo axes of a kabuli type, while Ganguly and co-workers reached 8.6% with a half-seed method [8, 9]. The superiority of cotyledonary nodes likely reflects their higher density of competent cells at the axillary meristem, which are receptive to Agrobacterium attachment and T-DNA transfer [10].

The inverse relationship between copy number and expression in line JG-T12 fits the well-known pattern of repeat-induced gene silencing [6]. Single-copy integrants generally express transgenes more stably, which has practical value for breeding programmes needing consistent expression across generations.

Larval mortality at 96 hours in the best line (78.3%) falls within the range seen in Bt cotton at comparable protein levels [4]. However, detached-leaf bioassays don't fully replicate field conditions. Whole-plant cage assays and confined field trials will be needed before these lines can advance toward regulatory evaluation. The ELISA-measured Cry1Ac levels (0.09-0.34 $\mu\text{g/g}$ FW) are modest compared to commercial Bt cotton events, which often reach 1-3 $\mu\text{g/g}$. Switching to a stronger or tissue-specific promoter, such as the chickpea pod-wall promoter described by Singh *et al.* [12], or stacking cry1Ac with cry2Ab, may improve performance.

Conclusion

This research confirmed that Agrobacterium-mediated transformation of chickpea with synthetic cry1Ac is feasible at efficiencies sufficient to generate a usable pool of transgenic events. Cotyledonary nodes from cultivar JG-62 gave 14.7% efficiency, significantly higher than the four other explant types. PCR and Southern blot verified stable integration of one to three copies in T₀ plants, and ELISA detected Cry1Ac at 0.09-0.34 $\mu\text{g/g}$ fresh weight.

The best transgenic line caused 78.3% neonate *H. armigera* mortality within 96 hours on detached leaves. Single-copy lines expressed higher and more consistent protein levels

than multi-copy lines. Going forward, T1 and T2 progeny analyses, whole-plant cage trials, and confined field experiments are needed to assess whether this protection holds under natural pest pressure. Combining cry1Ac with cry2Ab could widen the toxin spectrum and delay resistance evolution in *H. armigera*.

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